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(54) Title: DIAGNOSIS OF THROMBOTIC EVENTS BY DETECTING P–SELECTIN

(57) Abstract

The invention encompasses a method for determining the presence or absence of a thrombotic event by assessing membrane bound and/or soluble P–selectin. This dual assay measurement, called the P–selectin profile, allows a physician to assess platelet activation in a patient sample. The P–selectin profile also allows a physician to differentiate between patients who have a thrombotic disorder and those who are symptomatic but do not have a thrombotic disorder. The claimed invention also involves the assessment of a platelet activation marker and a thrombin generation marker and/or a diagnostic test to increase sensitivity in determining the presence or absence of a thrombotic event. The invention additionally provides an apparatus and a kit for measuring the P–selectin profile.

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DIAGNOSIS OF THROMBOTIC EVENTS BY DETECTING P-SELECTIN

RELATED APPLICATIONS

This application is a continuation-in-part and claims priority to U.S. Application No. 08/946,350, filed October 7, 1997, the teachings of which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

A thrombotic event occurs when a clot forms and lodges within a blood vessel. The blockage may fully block or partially block the blood vessel causing a thrombotic disorder such as a heart attack or stroke. Forms of a heart attack include angina and myocardial infarction.

Two phases of thrombotic events may exist, an ischemic stage and a necrotic stage. A patient may suffer from ischemia in which a decrease of blood flow may occur. This decrease in blood flow causes a decrease in tissue oxygenation. After prolonged ischemia, the tissue may undergo necrosis which is death of the tissue. Current methods for diagnosing thrombotic events can be time consuming. Thus, a need exists to quickly diagnose the presence or absence of an ischemic inducing thrombotic event to prevent tissue death or necrosis.

A need also exists to diagnose patients who may or may not be suffering from a thrombotic event. Often, a physician is faced with an emergency room patient complaining of symptoms such as chest pain which could point to several diagnoses. A patient who has chest pain could be suffering from a thrombotic event, such as a heart attack, or from something less serious, such as indigestion. Current techniques for detecting a thrombotic event may not be completely reliable. Therefore, a need exists to differentiate more accurately and quickly between a patient that is suffering from a thrombotic event and a patient that is not suffering from a thrombotic event.

SUMMARY OF THE INVENTION

The present invention relates to a method of diagnosing the presence or absence of a thrombotic disorder. A thrombotic event activates platelets, and therefore, monitoring platelet activation allows a physician to diagnose a thrombotic event. This method involves assessing the level of a sensitive marker for platelet activation in a patient sample, namely the marker, P-selectin.

Two forms of P-selectin exist. One form is soluble P-selectin and the other is membrane bound P-selectin. The claimed invention also allows a physician to diagnose a thrombotic event, such as a heart attack, by assessing the level of soluble and/or membrane bound P-selectin.

Assessment of soluble and membrane bound P-selectin is called a P-selectin profile or dual assay. Elevated levels of either or both indicate the presence of a thrombotic disorder in the patient.

Another embodiment of the invention is a method for differentiating between a thrombotic disorder and a symptomatic, but non-thrombotic disorder by assessing both soluble and membrane bound P-selectin. Such an assessment allows a physician to distinguish between patients suffering from a thrombotic event and those who are not.

The dual assay can also be used for a method of determining a patient's endogenous platelet activation. Elevated levels of either forms of P-selectin can be compared to a fully activated patient sample to determine the amount of endogenous platelet activation.

Another embodiment pertains to assessing the level of at least one platelet activation marker (e.g., soluble P-selectin, membrane bound P-selectin, or both) and the level of a thrombin generation marker (e.g., creatine kinase muscle brain, D-Dimer, F1.2, thrombin anti-thrombin, soluble fibrin monomer, fibrin peptide A, myoglobin, thrombin precursor protein, platelet monocyte aggregate or troponin). A still further embodiment comprises assessing the result of a diagnostic test (e.g., an electrocardiogram). The assessment of platelet activation, thrombin generation and/or a thrombotic diagnostic test increases the sensitivity of a thrombotic disorder

diagnosis and allows one to diagnose a thrombotic disorder closer to its onset.

The invention also relates to an apparatus or machine that is capable of measuring both levels of P-selectin, soluble and membrane bound. This apparatus has a means for detecting the level of membrane bound P-selectin and a means for detecting the level of soluble P-selectin. This apparatus may have a means for comparing the levels of P-selectin in a sample to normal levels to obtain a result. This machine can also have a means for displaying the results of the diagnosis.

Another aspect of the invention relates to a kit for use in diagnosing the presence or absence of a thrombotic disorder. The kit enables one to assay the levels of soluble P-selectin, membrane bound P-selectin and/or the number of platelets. The kit has one or more reagents for detecting the level of membrane bound P-selectin and one or more reagents for detecting soluble P-selectin.

The invention offers several advantages. For example, the invention allows a physician to more efficiently and quickly diagnose a patient who may have a thrombotic disorder. Furthermore, the invention enables a physician to quickly diagnose a thrombotic event and administer therapy promptly which could possibly prevent severe tissue necrosis or tissue death. The invention further provides a sensitive marker for platelet activation which allows for diagnosing and monitoring of a thrombotic disorder.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying figures. The emphasis of the figures is on illustrating the invention's principles.

Figure 1 is a schematic depiction illustrating a flow cytometry assay useful for the determination of membrane bound P-selectin. The following abbreviations are used in the figure which designate the various reagents utilized in the assay: ACD, Acid citrate dextrose; PGE_1 , prostaglandin E_1 ; PRP, platelet-rich plasma; A/P, apyrase plus prostaglandin E_1; MTB, modified Tyrodes Buffer; FITC, fluorescein isothiocyanate, PMA, phorbol 12-myristate 13-acetate, S12-FITC, FITC-labeled anti-P-selectin monoclonal antibody (Mab) S12.

Figure 2 is a schematic depiction illustrating a radioimmunoassay useful for the determination of membrane bound P-selectin. The following abbreviations are used in the figure which designate the various reagents utilized in the assay: ACD, Acid citrate dextrose; PGE_1, prostaglandin E_1; PRP, platelet-rich plasma; PPP, platelet poor plasma; PMA, phorbol myristate acetate; ^{125}I-S12 IgG, ^{125}I-labeled anti-P-selectin monoclonal antibody (Mab) S12.

Figure 3 is a schematic depiction illustrating a volumetric capillary cytometry system useful for the

determination of membrane bound P-selectin. The volumetric capillary cytometry system is called IMAGN2000™, from Biometric Imaging, Mountain View, CA. The following abbreviations are used in the figure which designate the various reagents utilized in the assay: ACD, Acid citrate dextrose; PGE_1 , prostaglandin E_1 ; WB, whole blood; A/P, apyrase plus prostaglandin E_1; MTB, modified Tyrodes Buffer; CD61-Cy5, Cy5-labeled Mab that binds a receptor found on essentially all platelets; S12/W40-Cy5, a mixture of equal parts of Cy5-labeled anti-P-selectin Mabs S12 and W40.

Figure 4 is a schematic depiction illustrating a volumetric capillary cytometry system useful for the determination of soluble P-selectin in plasma from a patient sample. The following abbreviations are used in the figure which designate the various reagents utilized in the assay: ACD, acid citrate dextrose, A/P, apyrase plus prostaglandin E_1 PPP, platelet poor plasma, W40, P-selectin specific Mab used to coat 9.7μM polystyrene particles; S12-Cy5, Cy5-labeled P-selectin specific Mab.

Figure 5 is a standard curve generated using the soluble P-selectin ELISA described in Example 2 with increasing concentrations (3.2 ng/ml to 320 ng/ml) of recombinantly produced, truncated P-selectin purified from tissue culture supernatant of human 293 transfectants. The assay format used a W40-coated microtiter plate. The standard was added to the plate simultaneously with

biotinylated S12 antibody and streptavidin-HRP, and incubated for 2 hours. Color development in the presence of OPD was stopped after 20 minutes with 4N H_2SO_4. A correlation coefficient of 0.996 or better was achieved. A log-log fit was chosen as best fit for the data. Inter- and intra-assay precision for the assay is CV < 10%.

Figures 6A-6B are graphs which depict the dose-dependent increase in platelet membrane P-selectin expression determined using a radioimmunoassay (RIA, Example 1). Figure 6A shows the dose-dependent increase in the binding of ^{125}I-S12 to platelets activated by PMA ranging from 5 - 500 nM final concentration. Figure 6B depicts the activation indices for platelet P selectin expression for a dose titration of PMA. The activation indices are the ratio between the endogenous P-selectin expressed and the P-selectin that could be expressed under conditions designed to stimulate expression of all available P-selectin.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of diagnosing the presence or absence of a thrombotic disorder by measuring the level of membrane bound P-selectin and/or soluble P-selectin. Elevated levels of either membrane bound P-selectin or soluble P-selectin, or both, indicate the presence of a thrombotic disorder. A physician can use the present invention to diagnose thrombotic disorders such

as myocardial infarction, unstable angina, stroke, pulmonary embolism, transient ischemic attack, deep vein thrombosis, and thrombotic re-occlusion. A thrombotic re-occlusion can occur subsequent to a coronary intervention procedure such as angioplasty or thrombolytic therapy.

Role of Platelets and P-selectin

Platelets play a key role in thrombotic disorders including arterial thrombosis and coronary syndromes (Gawaz, M. *et al.*, "Platelet function in acute myocardial infarction tested with direct angioplasty," *Circulation, 93*: 229-237 (1996); Trip, M.D. *et al.*, "Platelet hyperreactivity and prognosis in survivors of myocardial infarction," *N. Engl. J. Med., 322*: 1549-1554 (1990); Hirsh, J., "Hyperreactive platelets and complications of coronary artery disease," *N. Engl. J. Med., 316*: 1543-1544 (1989); Frink, R.J. *et al.*, "Coronary thrombosis and platelet/fibrin microemboli in death associated with acute myocardial infarction," *Br. Heart. J., 59*: 196-200 (1988)). Platelets become activated through numerous stimuli including thrombin, subendothelial interactions, contact with artificial surfaces, and in the presence of some immune complexes (Bellon, J.L. *et al.*, "Measurement of beta-thromboglobulin and platelet factor 4 to follow up patients with artificial heart valves," *Sem. in Thromb. and Hemost., 19(Suppl 1)*: 178-182 (1993); Scharf, R.E. *et al.*, "Activation of platelets in blood perfusing angioplasty-

damage coronary arteries: Flow cytometric detection," *Arteriosclerosis and Thrombosis, 12*: 1475-1487 (1992)). Once activated, platelets expose the fibrinogen binding sites on the membrane glycoprotein GP IIb/IIIa complex and platelet aggregation takes place through fibrinogen bridging thereby allowing a fibrin clot to form. (McEver, R.P., "The clinical significance of platelet membrane glycoproteins," *Hematol. Oncol. Clin. North Am., 4*: 87-103 (1990); Du, X. *et al.*, "Ligands "activate" integrin α_{IIb} β_3 (Platelet GP IIB-IIIa)," *Cell, 65*: 409-416 (1991)).

P-selectin, also known as CD62, GMP-140, or PADGEM, is a member of the selectin family of adhesion receptors (Lawrence, M.B. and T.A. Springer, *Cell, 65*: 859 (1991); Johnston, G.I. *et al., Cell, 56*: 1033-1044 (1989); McEver, R.P., U.S. Patent No. 5,378,464). P-selectin is an integral membrane glycoprotein found in the α granules of non-activated platelets and in the Weibel-Palade bodies of endothelial cells(Peerschke, E.I.B., *Am. J. Clin. Pathol., 98*: 455 (1992); McEver, R.P., 1993, "Leukocyte interactions mediated by P selectin," In: *Structure, Function and Regulation of Molecules Involved in Leukocyte adhesion,* Lipsky, P.E. *et al.*, Eds., (Springer Verlag, New York) pp. 135-150). Upon platelet activation, platelets degranulate and glycoproteins such as P-selectin diffuse out onto the surface where they can be detected with specific antibodies (Nurden, A.T. *et al., Nouv. Rev. Fr. Hematol., 35*: 67 (1993)).

Membrane bound P-selectin mediates the adherence of degranulated platelets to leukocytes in vivo (Rinder, H.M., et al., "Progressive platelet activation with storage: evidence for shortened survival of activated platelets after transfusion," *Transfusion* 31: 409-414 (1991)). Skilled artisans disagree about whether degranualated platelets are cleared from circulation or continue to circulate after platelet activation (Rinder, H.M., et al. "Progressive platelet activation with storage: evidence for shortened survival of activated platelets after transfusion." *Transfusion* 31: 409-414 (1991); Itoh, T., et al, "Can the risk for acute cardiac event in acute coronary syndrome be indicated by platelet membrane activation marker P selectin?" *Cor. Artery Dis.* 6: 645-650 (1995)). Therefore, diagnostic and clinical uses of membrane bound P-selectin have been unclear.

Skilled artisans also disagree about the source and function of soluble P-selectin. Studies reveal that the source may be activated platelets or endothelial cells or both, and consequently, the relationship between soluble P-selectin and platelet activation has been unclear (Michelson, A.D., et al.,*Proc. Natl. Acad., Sci. USA* 93: 11877-11882; Blann, A.D., et al. *Thromb. Haemost* 77: 1077-1080); Chong et al , *Blood* 83: 1535-1541).

Definitions

The term "P-selectin" includes P-selectin molecules such as mature protein (e.g., of platelet origin, of endothelial origin, membrane-bound, soluble), polymorphic or allelic variants of P-selectin, and other isoforms (e.g., produced by alternative splicing or other cellular processes), and modified or unmodified forms of the foregoing (e.g., glycosylated, unglycosylated). The term P-selectin also encompasses its soluble form which is produced by proteolytic cleavage of the membrane form from activated platelets.

For purposes of the claimed invention, the term, "antibody," encompasses polyclonal antibodies, monoclonal antibodies, single chain antibodies, chimeric, humanized, primatized, CDR-grafted, and veneered antibodies. The term antibody further includes portions derived from different species, human antibodies which are native or derived from combinatorial libraries, and the like. Conventional techniques can chemically join together the various portions of these antibodies. Genetic engineering techniques can also prepare the antibody as a contiguous protein. For example, nucleic acids encoding a chimeric or humanized chain can be expressed to produce a contiguous protein. See, e.g., Cabilly *et al.*, U.S. Patent No. 4,816,567; Cabilly *et al.*, European Patent No. 0,125,023 B1; Boss *et al.*, U.S. Patent No. 4,816,397; Boss *et al.*, European Patent No. 0,120,694 B1; Neuberger, M.S. *et al.*, WO 86/01533; Neuberger, M.S. *et al.*, European Patent No.

0,194,276 B1; Winter, U.S. Patent No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Queen *et al.*, European Patent No. 0 451 216 B1; and Padlan, E.A. *et al.*, EP 0 519 596 A1. See also, Newman, R. *et al.*, *BioTechnology, 10:* 1455-1460 (1992), regarding primatized antibody, and Ladner *et al.*, U.S. Patent No. 4,946,778 and Bird, R.E. *et al.*, *Science, 242:* 423-426 (1988)) regarding single chain antibodies.

In addition, functional fragments of antibodies, including fragments of chimeric, humanized, primatized, veneered or single chain antibodies can also be produced. Functional fragments of the foregoing antibodies include those which are reactive with P-selectin. For example, the invention encompasses antibody fragments capable of binding to P-selectin or portion thereof, including, but not limited to, Fv, Fab, Fab' and $F(ab')_2$ fragments. Enzymatic cleavage or recombinant techniques can produce these functional fragments. For instance, papain or pepsin cleavage can generate Fab or $F(ab')_2$ fragments, respectively. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons has been introduced upstream of the natural stop site. For example, a chimeric gene encoding a $F(ab')_2$ heavy chain portion can include DNA sequences encoding the CH_1 domain and hinge region of the heavy chain.

The term "antibody" also includes various forms of modified antibodies. For example, modification may occur by incorporating or attaching, directly or indirectly, (e.g., via a linker) a detectable label. The detectable labels may include a radioisotope, spin label, antigen label such as a FLAG tag, enzyme label, fluorescent or chemiluminescent group and the like.

The term "sample" means tissue, fluid, whole blood, plasma, serum and aqueous blood components, etc., from a patient. The term, sample, also includes any type of bodily substance containing activated platelets or any form of P-selectin.

The term "technician," "healthcare provider" or "researcher" refers to any person qualified or capable of obtaining a suitable sample and/or assessing the levels of P-selectin. These terms also encompass a person capable of comparing levels of P-selectin to those within normal limits to determine whether the sample levels of P-selectin are elevated. These terms are interchangeable.

The term "dual assay" or "P-selectin profile" means an assay capable of determining the levels of soluble and membrane bound P-selectin. In an assay measuring both levels, an elevated level of either can indicate the presence of a thrombotic event or platelet activation. The terms, "marker" or "marker for platelet activation" refer to either the level of soluble or the level of membrane bound P-selectin.

Diagnostic Applications

The invention embodies the diagnostic utility of soluble P-selectin and membrane bound (platelet) P-selectin, separately. Each form of P-selectin, individually, can be an indicator of a thrombotic disorder. Elevated levels of either form can indicate platelet activation, a thrombotic event and/or an impending thrombotic event.

However, analysis for both forms of P-selectin provides a more reliable indicator of thrombotic disorders. Surprisingly, it has been discovered that soluble and membrane bound P-selectin levels are each elevated at different times during the course of a thrombotic episode. Therefore, assessing both levels allows physicians to more accurately diagnose a thrombotic episode and avoid potentially false negative results.

Table 1 shows the increased diagnostic information obtained from the use of a P-selectin profile consisting of both the platelet and the soluble P-selectin determinations compared with either measurement alone. The patients shown in Table 1 all underwent balloon angioplasty (PTCA). For this patient group, platelet P-selectin level $\geq$ 5.7 percent positive was significantly elevated, and soluble P-selectin level $\geq$40 ng/ml was significantly elevated. For example, patient #1, the platelet P-selectin showed significant elevation prior to the PTCA procedure, but the soluble P-selectin was not indicative of abnormal platelet activation

at that time. Unexpectedly, the results for patient #2 are converse to those for patient #1. Unlike Patient #1, Patient #2's percent positive platelets prior to the angioplasty measured by platelet bound P-selectin was in the normal range while Patient #2's soluble P-selectin was, however, significantly elevated at that time. Therefore, the dual assay or P-selectin profile is a more reliable indicator of platelet activation and thrombotic disorders. The physician who treated patient #2, having knowledge of this patient's P-selectin profile, may have opted for a different course of treatment instead of using rotational atherectomy and/or the administering an anti-platelet therapy.

Table 1. Increase diagnostic sensitivity using the P-selectin Profile

Patient No.	Time relative to PTCA	Clinical Info	% P-selectin positive platelets	Soluble P-selectin (ng/ml)
1	Pre	ReoPro® anti platelet therapy	7.09	26.9
	Post		5.14	22.7
	24 hours		5.45	20.8
2	Pre	Rotational atherectomy	4.84	44.9
	Post		6.91	51.5
3	Pre(1)		3.81	
	Pre(2) after catheter insertion		4.26	14.21
	Post(stent 1)	ReoPro® anti platelet therapy	9.82	13.10
	Post(stent 2)		4.09	14.84
	24 hours		3.0	17.52

Table 2 contains data from twenty-three patients who had acute myocardial infarction (AMI) as defined by ST wave elevations. All of these patients were negative for troponin T at the time of diagnosis. Platelet P-selectin was measured flow cytometrically, as described herein, for these patients and for a group of control volunteers who did not have acute coronary syndrome. The ELISA method, as described herein, measured soluble P-selectin in the plasma of these 23 patients and a group of control volunteers who did not have acute coronary syndrome. A "+" symbol indicates a significantly elevated level of soluble and/or membrane bound P-selectin, and a "0" symbol indicates that the marker was not significantly elevated. Results in this table show the increased diagnostic sensitivity obtained by the determination and evaluation of the dual markers, platelet (membrane) bound and soluble P-selectin. These two markers comprise a P-selectin "profile". As seen in Table 2, soluble P-selectin determinations alone were elevated in 87% of the patients who had suffered an AMI. Platelet P-selectin alone would have detected 70% of these patients. The P-selectin "profile" which is positive if either marker of P-selectin is significantly elevated had a sensitivity of 95% in detecting this population of AMI patients. Table 2 illustrates the importance and surprising discovery of measuring both forms of P-selectin. A physician measuring

only one of the P-selectin forms would have obtained eight additional false negative results which means approximately 34% of the patients could have been mis-diagnosed. Unexpectedly, utilizing the dual assay, only a single patient, less than 5% of the patients, had a false negative result. This single false negative is believed to have occurred because the patient had exhausted platelet syndrome.

Table 2: INCREASED SENSITIVITY OF P-selectin PROFILE IN AMI DIAGNOSIS

Patient No	Platelet P-Selectin	Soluble P-Selectin	Profile Result
1	+	+	+
2	+	0	+
3	+	+	+
4	0	+	+
5	+	+	+
6	+	+	+
7	+	+	+
8	0	+	+
9	+	0	+
10	+	+	+
11	0	0	0
12	+	+	+
13	+	+	+
14	+	+	+
15	+	+	+
16	+	+	+
17	0	+	+
18	0	+	+
19	+	+	+
20	0	+	+
21	+	+	+
22	0	+	+
23	+	+	+

+ for soluble P-selectin = control mean + 2SD; + for platelet P-selectin = control mean + 1 SD; Patient #11 was an 86 year old man with "exhausted" platelets. He suffered a hemorrhagic stroke 20 hours after thrombolytic therapy.

The invention embodies a method for differentiating between a patient having a thrombotic disorder and a patient who presents similar symptoms, but does not have a thrombotic disorder. This type of assessment is common in a hospital emergency room. A quick and efficient screening test will enable physicians to more easily make this determination and diagnose the patient. The P-selectin profile or dual assay enables a physician to more easily distinguish between patients with and without a thrombotic disorder.

The invention utilizes an underlying concept which the inventors discovered. This concept deals with the temporal effect of platelet activation on P-selectin levels. The levels of soluble and membrane bound P-selectin each rise and fall at different times or stages throughout a thrombotic episode or disorder. The invention embodies and takes advantage of this concept by measuring *both* forms of P-selectin to diagnose the occurrence of a thrombotic event or disorder.

Accordingly, Table 3 illustrates the important utility for a method of screening or differentiating between patients with and without a coronary disorder using the P-selectin profile. Table 3 shows the results of platelet and soluble P-selectin determinations for 8 patients who entered the emergency department with a diagnosis of either unstable angina(UA) or AMI. In this table, a "+" indicates significant elevation while a "0" indicates that no significant elevation was observed. This table clearly

shows that measurement of both platelet and soluble P-selectin increases the diagnostic sensitivity of P-selectin detection as a marker of platelet activation. Patients #5002, 5003, 5006 showed insignificant elevations of their soluble P-selectin, but significant elevations of platelet bound P-selectin. By contrast, patient #5009 at 6 hours after admission to the emergency department was negative for platelet bound P-selectin, but showed significant elevation in the soluble P-selectin assay. This result illustrates an important advantage of the P-selectin profile. The patients in Table 2 appeared in the emergency room on an average of 4.5 hours after the onset of chest pain and 87% of these patients had elevated soluble P-selectin while 70% were positive for platelet P-selectin. By contrast, the patients shown in Table 3 appeared in the emergency room an average of 2.5 hours after the onset of chest pain and while only 2 out of 8 were positive for soluble P-selectin, and 6 out of 8 were positive for platelet P-selectin. These results point to the important temporal link between the onset of platelet activation and the appearance of the platelet activation marker, P-selectin, on the platelet membrane or in the plasma. Platelets express platelet P-selectin immediately upon activation, but they may cleave from the surface of circulating platelets within 2 hours of expression. If the soluble P-selectin elevation observed depends on the cleavage of P-selectin from the surface of activated platelets and/or the production of a soluble form of P-

selectin by activated platelets, then some time period must pass after the initiation of platelet activation before a significantly elevated level of soluble P-selectin occurs. The exact window required for this to occur is likely to be specific to each individual. The measurement of only one form of P-selectin is not as sensitive as a diagnostic tool as is the sensitivity of the dual assay. The dual assay allows a physician to detect or rule out acute coronary syndrome because the expression of platelet P-selectin after activation has a narrow window and the appearance of soluble P-selectin is dependent on the passage of time after platelet activation. The combined measurement or P-selectin profile eliminates the possibility that detection of platelet activation will not occur even though time may have passed after the thrombotic event.

Table 3: MCV P-selectin PROFILE STUDY

Patient #	Sample Time Hours	Diagnosis	Platelet P-selectin (% postive)	Soluble P-selectin	Profile	CKMB (ng/ml)	Troponin 1 (ng/ml)
5002	0	UA/MI	7.3(+)	0	+	6.14	0.58
5002	3		4.57(+)	0		6.99	0.55
5003	0	UA	19.08(+)	0	+		
5003	3		14.56(+)	0		<0.6	<0.5
5004	0	UA	5.15(+)	0	+	0.6	<0.5
5004	3		7.29(+)	0			
5005	0	UA	7.16(+)	+	+	1.7	<0.5
5005	3		8.93(+)	+		1.4	
5006	0	UA	3.89(+)	0	+	<0.6	<0.5
5006	3		7.80(+)	0		<0.6	<0.5
5007	0	UA	3.17(0)	0	0	0.7	<0.5
5007	3		2.21(0)	0			
5008	0	AMI	1.70(0)	0	0		
5008	3		1.22(0)	0			
5008	6		2.49(0)	0			
5009	0	UA/MI	11.38(+)	+	+	3.7	0.9
5009	3		3.75(+)	+		25.6	
5009	6		0.87(0)	+			

A preferred embodiment of the invention is to assess a platelet activation level (e.g., soluble P-selectin, membrane bound P-selectin, or both) in conjunction with at least one additional diagnostic test and/or marker of thrombin generation. A marker for thrombin generation is a protein or fragment thereof that is activated or expressed when the clotting cascade occurs and thrombin and/or fibrin is present. Such a marker include various end products that are present as a results of the thrombosis, including necrosis markers and/or factors. Examples of thrombin generation markers include, but are not limited to, Creatine Kinase with Muscle and/or Brain subunits (CKMB), D-Dimer, F1.2, thrombin anti-thrombin (TAT), soluble fibrin monomer (SFM), fibrin peptide A (FPA), myoglobin, thrombin precursor protein (TPP), platelet monocyte aggregate (PMA) and troponin. Assessing a platelet activation marker (e.g., soluble and/or platelet P-selectin) is sensitive, by itself, as described herein. However, assessing levels of both a platelet activation marker and a thrombin generation marker significantly increases sensitivity, and does so earlier to the onset of a thrombotic event. A positive result in either the platelet activation marker and/or the thrombin generation marker means that the patient is undergoing a thrombotic event and allows one to accurately diagnose more patients that would have otherwise been missed.

This additive effect of assessing the results of a platelet activation marker and a thrombin generation marker

stems from the biological processes of platelet activation and clot formation. A thrombin generation marker refers to assessing the level of clot formation, thrombin formation, or fibrin formation. When a person undergoes a thrombotic event, platelet activation occurs and a clot begins to form via the clotting cascade. A clot forms when thrombin is activated, which, in turn, activates fibrinogen to make fibrin. Activated platelets often get caught and become part of the clot. The clot composition varies and can comprise a higher percentage of either platelets or fibrin. The composition of the clot depends on many factors, including the amount of time the clot has been formed. Accordingly, monitoring both pieces of the process, e.g., the platelet activation and thrombin/fibrin formation, more accurately assesses patients undergoing a thrombotic event.

Combining the assessment of a platelet activation marker with the assessment of results for a diagnostic test also increases sensitivity of determining the presence or absence of a thrombotic event. The diagnostic test is a one that can assess the presence or absence of a thrombotic disorder. The diagnostic test can be a currently known or utilized test (e.g., an electrocardiogram) or one later developed. Assessing all three components, e.g., assessing platelet activation, thrombin generation, and a diagnostic test, significantly increases the accuracy of a thrombotic disorder diagnosis, as shown and described herein.

The combination of platelet activation assessment with either a thrombin generation assessment or a thrombotic

diagnostic test allows one to make a diagnosis earlier. A thrombotic disorder can be diagnosed from its onset (time point of zero) up to about 24-48 hours. Preferably, the diagnosis occurs within 6 or 8 hours of onset or presentation (e.g., before 1,2, or 3 hours).

A clinical feasibility study of 600 patients was performed which demonstrated that the P-selectin profile is a successful tool in diagnosing patients who are undergoing a thrombotic disorder. The patients who complained of chest pain at the emergency rooms from various area hospitals (Cleveland Clinic, Univ. of Cincinnati, Thomas Jefferson Univ. Hospital, Sinai Hospital - Baltimore, University of Pennsylvania Hospital) were screened for soluble and membrane bound P-selectin. The patients were also given an ECG (electrocardiogram, and screened for CKMB (Creatine Kinase with Muscle and/or Brain subunits).

The levels of soluble and membrane bound P-selectin were compared against the patient's diagnosis and standard methods of diagnosing a cardiac, thrombotic disorder. Tests for soluble and platelet P-selectin were performed by operators with no knowledge of the diagnosis (Acute myocardial infarction, Unstable angina, Chest pain of unknown etiology/other). Tests for soluble and platelet P-selectin were positive if the value for that marker was 2 standard deviations above the mean obtained for the 200 normal donors tested. The value of 2 standard deviations above the mean puts the test in the 95% confidence interval meaning that 95 out of 100 normal donors will be negative

using this cut-off or limit. Tests for soluble and platelet P-selectin were done at admission and in some cases additional tests were performed at 1, 2, and 6 hours after presentation. See Table 4. The abbreviations for Table 4 are as follows: MI= Myocardial Infarction; UA= Unstable Angina; CPUE=Chest Pain of Unknown Etiology; ECG= Electrocardiogram; P+ECG= Profile + Electrocardiogram results; P+CKMB= Profile + CKMB results; ECG+CKMB= Electrocardiogra + CKMB results; P+E+CKMB= Profile + ECG + CKMB results; Prior AMI = Prior Acute Myocardial Infarction.

Table 4. P-Selectin Clinical Trial Data Analysis

		P-SELECTIN										
DIAG	Count	Bound	Soluble	Profile	ECG	CKMB	P+ECG	P+ CKMB	ECG+ CKMB	P+E+ CKMB	Diabetes	Prior AMI
MI	58	29	18	35	27	31	45	49	44	53	13	18
%		50.0	31.0	60.3	46.6	53.4	77.6	84.5	75.9	91.4	22.4	31.0
UA	98	44	17	51	8	2	54	53	10	56	29	46
%		44.9	17.3	52.0	8.2	2.0	55.1	54.1	10.2	57.1	29.6	46.9
CPUE	151	48	18	60	6	0	62	60	6	62	27	26
%		31.8	11.9	39.7	4.0	0.0	41.1	39.7	4.0	41.1	17.9	17.2
Other	88	35	14	42	2	0	43	42	2	43	12	18
%		39.8	15.9	47.7	2.3	0.0	48.9	47.7	2.3	48.9	13.6	20.5
Subtotal	395	156	67	188	43	33	204	204	62	214	81	108
		39.5	17.0	47.6	10.9	8.4	51.6	51.6	15.7	54.2	20.5	27.3
Missing	205	86	37	100	3	3	101	100	5	101	23	18
%		42.0	18.0	48.8	1.5	1.5	49.3	48.8	2.4	49.3	11.2	8.8
Total	600	242	104	288	46	36	305	304	67	315	104	126
		40.3	17.3	48.0	7.7	6.0	50.8	50.7	11.2	52.5	17.3	21.0

Summary statistics, shown herein, are based on the data obtained at presentation. The most advantageous time to determine when a patient is undergoing a thrombotic event is when the patent presents the symptoms. The P-selectin profile differentiates chest pain of cardiac origin from non-cardiac based pain and allows for earlier diagnosis and proper treatment.

The current standard practices for screening a patient who presents chest pain include a CKMB test, ECG, and physical examination. At presentation, the results of a ECG reveals that a patient is undergoing an AMI in fewer than half of the cases. Likewise, the CKMB is positive in fewer than half of the AMI patients at presentation. The theory is the P-selectin, as a marker of ischemia, will be positive before CKMB, which is a marker of necrosis and only becomes a positive after muscle damage to the heart has occurred. From the data in Table 4, 60% of the AMI patients were positive for the P-selectin profile at presentation. This is more than for either the CKMB test or the ECG test alone.

A preferred embodiment is to utilize the P-selectin profile with other cardiac, diagnostic tools to obtain an earlier, more accurate diagnosis. The significant utility of the P-selectin profile is shown when the patients who are positive for the P-selectin profile are added to those positive for ECG and CKMB. Combining the results of these tests show that one can detect a high percentage (e.g., above 90%, in one study about 91.4%) of the AMI patients at

presentation. Combining the results from the ECG and CKMB show that they can diagnose 75.9% of the AMI patients at presentation. Therefore, an additional 15.5% (91.4% - 75.5%) of the patients having a *bona fide* AMI can be detected at presentation if the P-selectin profile is added to the diagnostic tests performed upon emergency department admission.

Tables 5 and 6 contain the data shown in Table 4, but sorted for patients diagnosed with myocardial infarction (MI). Sixty patients were diagnosed with MI. Over 90% of the patients diagnosed with MI were detected at presentation using a combination of the P-selectin profile, CKMB and ECG. The P-selectin profile detected over 80% of the patients who were undergoing an MI, compared with the 38.3% and 55.0% obtained from assessing the the CKMB level and ECG results, respectively. The P-selectin profile, by itself, is a good indicator of MI, and combined with other diagnostic tests, provides an even more precise indicator of MI.

Table 5

Patient Number	Bound P-Selectin	Soluble P-Selectin	Sum	P-selectin Profile	ECG	CKMB	Diabetes	Prior AMI
6	1	1	2	1	0	0	1	0
11	1	0	1	1	0	1	1	1
14	1	1	2	1	0	1	0	0
20	0	0	0	0	0	1	0	0
28	0	1	1	1	0	1	1	0
3	0	0	0	0	1	1	0	0
5	1	0	1	1	0	1	0	0
7	1	0	1	1	0	1	0	0
29	1	0	1	1	1	0	0	0
43	1	0	1	1	1	1	0	0
53	0	1	1	1	1	0	0	0
4	1	1	2	1	0	1	0	0
30	1	1	2	1	0	0	0	0
34	0	1	1	1	0	1	0	1
108	0	0	0	0	1	1	0	1
121	1	0	1	1	0	1	1	1
131	1	0	1	1	0	1	0	1
156	0	0	0	0	1	0	1	0
157	0	1	1	1	0	0	1	0
168	1	1	2	1	1	1	0	0
224	0	0	0	0	0	0	0	1
231	0	1	1	1	0	0	0	0

Patient Number	Bound P-Selectin	Soluble P-Selectin	Sum	P-selectin Profile	ECG	CKMB	Diabetes	Prior AMI
260	1	0	1	1	0	0	0	0
262	1	0	1	1	0	1	0	0
10	1	1	2	1	0	0	1	1
14	1	0	1	1	0	0	0	1
30	1	0	1	1	1	1	0	0
57	1	1	2	1	1	0	0	0
69	1	0	1	1	1	1	0	1
74	1	1	2	1	1	0	0	0
88	1	0	1	1	0	0	0	0
94	1	1	2	1	0	0	0	0
100	1	0	1	1	0	0	0	1
102	1	0	1	1	1	0	0	0
103	0	1	1	1	0	1	0	0
121	1	1	2	1	1	1	1	0
2	1	0	1	1	1	0	0	0
11	1	1	2	1	0	0	0	0
15	0	0	0	0	1	0	0	0
17	1	1	2	1	0	0	0	1
23	1	0	1	1	1	1	0	0
28	1	0	1	1	0	1	0	1
2	0	0	0	0	0	1	0	0
12	0	0	0	0	0	1	0	0
27	0	0	0	0	0	1	1	1

Patient Number	Bound P-Selectin	Soluble P-Selectin	Sum	P-selectin Profile	ECG	CKMB	Diabetes	Prior AMI
4	0	0	0	0	1	0	0	1
37	0	0	0	0	0	1	0	0
45	0	0	0	0	0	1	0	0
47	1	0	1	1	1	1	0	0
56	0	0	0	0	0	0	0	0
129	0	0	0	0	1	1	1	0
142	0	0	0	0	1	0	0	0
190	0	0	0	0	0	1	0	1
219	0	0	0	0	0	1	0	0
249	0	0	0	0	1	1	1	0
25	0	0	0	0	0	0	0	1
104	0	0	0	0	0	0	1	1
112	0	0	0	0	0	0	0	0
44	0	0	0	0	1	1	0	0
50	0	0	0	0	1	1	1	1
count=	31	18	49	37	23	33	13	18
	51.7%	30.0%	81.7%	61.7%	38.3%	55.0%	21.7%	30.0%

Total count=60 patients; 1= positive for the test (above the cut-off which was 2 standard deviations above the mean); 0=negative for the test (below the cut-off which was 2 standard deviations above the mean).

Table 6: P-selectin Clinical Trial Data Analysis

Attribute	Diagnosis	Count	Instrument	Time	P-selectin			ECG	CKMB	Time to	Diabetes	Prior
				Since Onset	Bound	Soluble	Profile			CKMB		AMI
ALL	MI	60	ALL		51.7	30.0	61.7	38.3	55.0		21.7	30.0
JEFF	MI	8	B		62.5	25.0	62.5	62.5	50.0		12.5	37.5
BALT	MI	14	B		78.6	42.9	85.7	42.9	28.6		21.4	35.7
HUP	MI	19	B		36.8	31.6	52.6	31.6	57.9		26.3	31.6
CINCY	MI	10	C		50.0	10.0	60.0	60.0	70.0		0.0	10.0
CLVND	MI	8	B		37.5	37.5	50.0	0.0	87.5		50.0	25.0
ECG Pos	MI	23	ALL		52.2	21.7	56.5	100.0	56.5		21.7	17.4
ECG Neg	MI	37	ALL		51.4	35.1	64.9	0.0	54.1		21.6	37.8
CKMB Pos	MI	33	ALL		48.5	21.2	57.6	39.4	100.0		24.2	30.3
CKMB Neg	MI	27	ALL		55.6	40.7	66.7	37.0	0.0		18.5	29.6
Diabetes Y	MI	13	ALL		38.5	38.5	53.8	38.5	61.5		100.0	46.2
Doabetes N	MI	47	ALL		55.3	27.7	63.8	38.3	53.2		0.0	25.5
PrioAMI Y	MI	18	ALL		50.0	16.7	55.6	22.2	55.6		33.3	100.0
PrioAMI N	MI	42	ALL		52.4	35.7	64.3	45.2	54.8		16.7	0.0

Attribute	Diagnosis	Count	Instrument	Time	P-selectin			ECG	CKMB	Time to	Diabetes	Prior
				Since Onset	Bound	Soluble	Profile			CKMB		AMI
Becton D	MI	49	n/a		53.1	34.7	63.3	34.7	53.1		26.5	32.7
Coulter	MI	11	n/a		45.5	9.1	54.5	54.5	63.6		0.0	18.2
Bound Pos	MI	31	ALL		100.0	38.7	100.0	38.7	51.6		16.1	29.0
Bound Neg	MI	29	ALL		0.0	20.7	20.7	37.9	58.6		27.6	31.0
Soluble Pos	MI	18	ALL		66.7	100.0	100.0	27.8	38.9		27.8	16.7
Soluble Neg	MI	42	ALL		45.2	0.0	45.2	42.9	61.9		19.0	35.7
Profile Pos	MI	37	ALL		83.8	48.6	100.0	35.1	51.4		18.9	27.0
Profile Neg	MI	23	ALL		0.0	0.0	0.0	43.5	60.9		26.1	34.8
High					78.6	42.9	85.7	62.5	87.5		50.0	37.5
Low					36.8	10.0	52.6	31.6	28.6		0.0	10.0
Median	MI	60	ALL		51.7	30.0	61.7	38.3	55.0		21.7	30.0

Table 7 shows that, in this study, 42.7% of patients whose chest pain is of unknown etiology also have a positive P-selectin profile. There are several reasons for this. The tests were not always performed in accord with the protocol. In particular, some of the tests for platelet P-selectin were not performed within the 30-minute window prescribed by the protocol. If blood is allowed to sit in a glass vacutainer tube for greater than 30 minutes, artificial elevation of the platelet P-selectin can occur. Also, the possibility exists that some of the emergency room diagnoses are incorrect. These are diagnoses that are not made by cardiologists, but by ER staff and perhaps some of the cases of chest pain of unknown etiology are actually cardiac events, which went undetected. In some instances, P-selectin could be detecting events that with the current diagnostic tests are not identified, and therefore, an incorrect ER diagnosis is given. P-selectin can simply be a victim of its own sensitivity which can have an adverse effect on its perceived specificity. Finally, it must be considered that platelet activation occurs in cases other than AMI and unstable angina and it may be that the P-selectin profile is detecting platelet activation occurring as a result of atrial fibrillation, sickle-cell disease or cancer.

Table 7. P-SELECTIN CLINICAL FEASIBILITY DATA SUMMARY						
All Corners Time "0"						
		P-selectin				
Diag	Count	Bound	Soluble	Profile	ECG	CKMB
MI	58	29	18	35	27	31
%		50.0	31.0	60.3	46.6	53.4
UA	98	44	17	51	8	2
%		44.9	17.3	52.0	8.2	2.0
CPUE/ OTHER	239	83	32	102	8	0
%		34.7	13.4	42.7	3.3	0.0
Total	395	156	67	188	43	33
%		39.5	17.0	47.6	10.9	8.4

Samples for normal donors were obtained from two sites and the data are shown in Table 8. The limit or cut off for a positive platelet or soluble P-selectin amount was determined by adding two standard deviations above the mean. The limit for soluble P-selectin is 154.9 ng/mL. The limit or cut off for platelet P-selectin varied depending on the type of flow cytometer used to measure it. The Becton Dickinson flow cytometer yielded a cut-off of 2.5%, whereas a Coulter flow cytometer revealed a cut off of 4.0% positive platelets. The soluble P-selecting level for these 600 patients and 200 normal donors were assessed using the methods described in Example 9. The platelet P-selectin was detected by flow cytometry using the method set forth in Example 10.

Table 8. P-SELECTIN REFERENCE RANGES FROM BOSTON AND SACRAMENTO

LOCATION	AGE GROUP	GENDER	ASSAY	N	MEAN	STD DEV	LIMIT
BOSTON	35-54	MALES AND FEMALES	SOLUBLE (ng/ml) PLATELET (%)	56 52	99.8 1.8	28.33 0.35	156.8 2.3
BOSTON	55 or older	MALES AND FEMALES	SOLUBLE (ng/ml) PLATELET (%)	35 34	107.4 1.5	30.39 0.41	168.1 2.6
BOSTON	ALL AGES	FEMALE	SOLUBLE (ng/ml) PLATELET (%)	43 41	97.0 1.7	30.03 0.38	157.1 2.5
BOSTON	ALL AGES	MALE	SOLUBLE (ng/ml) PLATELET (%)	46 45	108.3 1.7	27.86 0.38	163.4 2.6
BOSTON	ALL AGES	MALES AND FEMALES	SOLUBLE (ng/ml) PLATELET (%)	81 86	102.7 1.7	28.20 0.38	161.1 2.6
SACRAMENTO	35-54	MALES AND FEMALES	SOLUBLE (ng/ml) PLATELET (%)	51 43	97.7 2.4	27.99 0.71	153.7 3.8
SACRAMENTO	55 or older	MALES AND FEMALES	SOLUBLE (ng/ml) PLATELET (%)	49 45	95.7 2.6	24.09 0.71	143.9 4.1
SACRAMENTO	ALL AGES	FEMALE	SOLUBLE (ng/ml) PLATELET (%)	51 45	94.9 2.6	27.83 0.74	150.6 4.0
SACRAMENTO	ALL AGES	MALE	SOLUBLE (ng/ml) PLATELET (%)	49 45	98.6 2.5	24.16 0.70	147.0 3.9

LOCATION	AGE GROUP	GENDER	ASSAY	N	MEAN	STD DEV	LIMIT
SACRAMENTO	ALL AGES	MALES AND FEMALES	SOLUBLE (ng/ml) PLATELET (%)	100 91	96.7 2.5	28.04 0.72	148.8 4.0
BOTH SITES	35-54	MALES AND FEMALES	SOLUBLE (ng/ml) PLATELET (%)	107 96	98.8 2.0	28.05 0.66	154.9 3.3
BOTH SITES	55 or older	MALES AND FEMALES	SOLUBLE (ng/ml) PLATELET (%)	84 82	100.6 2.3	27.33 0.74	155.2 3.8
BOTH SITES	ALL AGES	FEMALE	SOLUBLE (ng/ml) PLATELET (%)	96 86	95.9 2.2	28.75 0.73	153.4 3.6
BOTH SITES	ALL AGES	MALE	SOLUBLE (ng/ml) PLATELET (%)	95 91	103.3 2.1	26.18 0.69	156.7 3.6
BOTH SITES	ALL AGES	MALES AND FEMALES	SOLUBLE (ng/ml) PLATELET (%)	181 177	99.6 2.1	27.68 0.71	154.9 3.5

OUTLIERS HAVE BEEN DELETED FROM ANALYSES

The invention also embodies an apparatus for measuring levels for both forms of P-selectin. In the preferred embodiment, the apparatus is an improved volumetric capillary cytometry system which is modified to measure P-selectin. The volumetric capillary cytometry system is known in the art and an embodiment is described in US patent No. 5,547,849, issued August 20, 1996 entitled, " Apparatus and Method for Volumetric Capillary Cytometry," the teachings of which are incorporated herein by reference in their entirety. Also, US patent No. 5,585,246, issued December 17, 1996, entitled, "Method for Preparing a Sample in a Scan Capillary for Immunofluorescent Interrogation," the teachings of which are incorporated herein by reference in their entirety, describes a method for fluorescent detection of surface antigens from white blood cells.

The apparatus comprises a means for detecting the level of soluble P-selectin as well as a means for detecting the level of membrane bound P-selectin. Detection of P-selectin can each occur in separate capillaries or chambers, or preferably, in one capillary or chamber. This machine will also have a means for displaying the results of this detection. One embodiment encompasses displaying the levels of P-selectin and allowing the technician or physician operating the machine to determine whether the levels are within normal ranges. More preferably, the apparatus further comprises a means for comparing the sample's levels of P-selectin with those within a normal

range and then displaying this result (ie, positive or negative for a thrombotic disorder).

Another aspect of the invention is a kit for determining the presence or absence of a thrombotic disorder. The kit comprises one or more reagents for measuring each of the P-selectin forms. The kit can be used in conjunction with the above described apparatus. The kit contains similar reagents for measuring both forms of P-selectin in the methods discussed in this document. For example, the kit can comprise reagents for detecting the level of membrane bound P-selectin which are similar as those used in the flow cytometry and volumetric capillary cytometry system methods. Therefore, reagents useful for measuring membrane bound and soluble P-selectin can include one or more of the following: antibodies specific to P-selectin, antibodies specific to a complex between P-selectin and an anti-P-selectin antibody. These antibodies can be detectably labeled as described in this document.

A preferred embodiment of the kit can also include a solid support. Examples of a solid supports are beads, a solid support strip and a modified capillary surface which may attach to the apparatus.

The kit can also comprise another one or more reagents used to measure a total platelet count. As discussed in this document, a total platelet count includes an antibody specific to a receptor on the platelet. The preferred embodiment utilizes an antibody specific to the glycoprotein GP IIb/IIIa, CD41, CD61, or CD42 receptors.

Immunological Assessment of P-Selectin

Methods that measure soluble and membrane bound P-selectin include several suitable assays. Suitable assays encompass immunological methods, such as FACS analysis, radioimmunoassay, flow cytometry, enzyme-linked immunosorbent assays (ELISA), chemiluminescence assays, and assessment with a volumetric capillary cytometry system. Any method known now or developed later can be used for measuring P-selectin.

The inventive methods utilize antibodies reactive with P-selectin or portions thereof. In a preferred embodiment, the antibodies specifically bind with membrane bound and/or soluble P-selectin or a portion thereof (see e.g., Furie *et al.*, U.S. Patent No. 4,783,330, the teachings of which are incorporated herein by reference in their entirety). The antibodies can be polyclonal or monoclonal, and the term antibody is intended to encompass polyclonal and monoclonal antibodies, and functional fragments thereof. The terms polyclonal and monoclonal refer to the degree of homogeneity of an antibody preparation, and are not intended to be limited to particular methods of production.

In several of the preferred embodiments, immunological techniques detect P-selectin levels by means of an anti-P-selectin antibody (i.e., one or more antibodies), such as monoclonal antibodies S12 or W40. The term "anti-P-selectin" antibody includes monoclonal and/or polyclonal antibodies, and mixtures thereof. For example, these immunological techniques can utilize mixtures of polyclonal and/or

monoclonal antibodies, such as a cocktail of murine W40, S12 and G1 monoclonal antibodies.

A researcher can raise anti-P-selectin antibodies against an appropriate immunogen, such as isolated and/or recombinant P-selectin or portion thereof (including synthetic molecules, such as synthetic peptides). In one embodiment, antibodies are raised against an isolated and/or recombinant P-selectin or portion thereof (e.g., a peptide) or against a host cell which expresses recombinant P-selectin (Johnston, G.I. *et al., Cell, 56*: 1033-1044 (1989); and McEver, R.P., U.S. Patent No. 5,378,464, the teachings of which are both incorporated herein by reference in their entirety). In addition, cells expressing recombinant P-selectin, such as transfected cells, can be used as immunogens or in a screen for antibody which binds receptor (See e.g., Chuntharapai *et al., J. Immunol., 152:* 1783-1789 (1994); Chuntharapai *et al.*, U.S. Patent No. 5,440,021).

Any suitable technique can prepare the immunizing antigen and produce polyclonal or monoclonal antibodies. The prior art contains a variety of these methods (see e.g., Kohler *et al., Nature, 256:* 495-497 (1975) and *Eur. J. Immunol. 6:* 511-519 (1976); Milstein *et al., Nature 266: 550-552 (1977);* Koprowski *et al.*, U.S. Patent No. 4,172,124; Harlow, E. and D. Lane, 1988, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY); *Current Protocols In Molecular Biology*, Vol. 2 (Supplement 27, Summer '94), Ausubel, F.M. *et al.*,

Eds., (John Wiley & Sons: New York, NY), Chapter 11, (1991)). Generally, fusing a suitable immortal or myeloma cell line, such as SP2/0, with antibody producing cells can produce a hybridoma. Animals immunized with the antigen of interest provide the antibody producing cell, preferably cells from the spleen or lymph nodes. Selective culture conditions isolate antibody producing hybridoma cells while limiting dilution techniques produce them. Researchers can use suitable assays such as ELISA to select antibody producing cells with the desired specificity.

Other suitable methods can produce or isolate antibodies of the requisite specificity. Examples of other methods include selecting recombinant antibody from a library or relying upon immunization of transgenic animals such as mice which are capable of producing a full repertoire of human antibodies (see e.g., Jakobovits *et al., Proc. Natl. Acad. Sci. USA, 90*: 2551-2555 (1993); Jakobovits *et al., Nature, 362*: 255-258 (1993); Lonberg *et al.*, U.S. Patent No. 5,545,806; Surani *et al.*, U.S. Patent No. 5,545,807).

According to the method, an assay can determine the level of P-selectin in a biological sample. In determining the amounts of membrane bound and/or soluble P-selectin, an assay includes combining the sample to be tested with an antibody having specificity for P-selectin, under conditions suitable for formation of a complex between antibody and P-selectin, and detecting or measuring (directly or indirectly) the formation of a complex. The

sample can be obtained directly or indirectly (e.g., provided by a healthcare provider), and can be prepared by a method suitable for the particular sample (e.g., whole blood, platelet rich plasma, platelet poor plasma, serum) and assay format selected. For example, suitable methods for whole blood collection are venipuncture or obtaining blood from an in-dwelling arterial line. The container into which a healthcare provider deposits the blood can contain an anti-coagulant such as ACD-A, heparin, or EDTA. Methods of combining sample and antibody, and methods of detecting complex formation are also selected to be compatible with the assay format. Suitable labels can be detected directly, such as radioactive, fluorescent or chemiluminescent labels. They can also be indirectly detected using labels such as enzyme labels and other antigenic or specific binding partners like biotin. Examples of such labels include fluorescent labels such as fluorescein, rhodamine, CY5, chemiluminescent labels such as luciferase, radioisotope labels such as ^{32}P, ^{125}I, ^{131}I, enzyme labels such as horseradish peroxidase, and alkaline phosphatase, β-galactosidase, biotin, avidin, spin labels and the like. The detection of antibodies in a complex can also be done immunologically with a second antibody which is then detected (e.g., by means of a label). Conventional methods or other suitable methods can directly or indirectly label an antibody.

Assaying for Detection of Membrane Bound P-selectin and Total Platelet Count

Any method known now or developed later can be used for measuring membrane bound P-selectin. One method for assessing membrane bound P-selectin levels which the invention utilizes is flow cytometry. Methods of flow cytometry for measuring platelet or membrane bound P-selectin are well know in the art. (Shattil, Sanford J, et al. "Detection of Activated Platelets in Whole Blood using Activation-Dependant Monoclonal Antibodies and Flow Cytometry," *Blood*, Vol. 70, No 1 (July), 1987: pp307-315; Scharf, Rudiger E., et al., "Activation of Platelets in Blood Perfusing Angioplasty-damaged Coronary Arteries, Flow Cytometric Detection," *Arteriosclerosis and Thrombosis*, Vol 12, No 12 (December), 1992: pp 1475-1487, the teachings of which are incorporated herein by reference in their entirety). Also, the teachings of co-pending application, serial number 08/748,387, filed November 13, 1996, entitled "Assessment of P-selectin in Venous Thrombotic Disorders, Vascular Interventions and Monitoring of Anti-Platelet Therapy," are incorporated herein by reference in their entirety.

For example, a sample comprising platelets can be contacted with an antibody having specificity for P-selectin under conditions suitable for formation of a complex between an antibody and P-selectin expressed on platelets, and detecting or measuring (directly or indirectly) the formation of a complex. In a particularly

preferred embodiment, the antibody, S-12 is conjugated with FITC. Figure 1 illustrates one type of flow cytometry assay. (see also Example 4).

For example, the level of membrane bound P-selectin can be assessed by flow cytometry comprising:

(a) obtaining a first and second sample comprising platelets,

(b) contacting said first sample, serving as a control, with a platelet activation agonist, such as phorbol myristate acetate (PMA), ADP (adenosine diphosphate), thrombin, collagen, and/or TRAP (thrombin receptor activating peptide), under conditions suitable for activation of platelets in said first sample, preferably for a period of time effective to maximally activate said platelets, and preferably while maintaining the second sample under conditions suitable for maintaining the endogenous platelet activation level;

(c) contacting or staining the samples with a composition comprising an anti-P-selectin antibody, such as an anti-P-selectin antibody comprising a fluorescent label, preferably in an amount in excess of that required to bind the P-selectin expressed on the platelets, under conditions suitable for the formation of labeled complexes between said anti-P-selectin antibody and activated platelets; and

(d) determining (detecting or measuring) the formation of complex in said samples, wherein the amount of complex detected indicates the extent of platelet activation in said second sample.

In addition to using flow cytometry to measure membrane bound P-selectin, a radioimmunoassay can also be employed and is fully described in co-pending application, serial number 08/748,387, filed November 13, 1996, entitled "Assessment of P-selectin in Venous Thrombotic Disorders, Vascular Interventions and Monitoring of Anti-Platelet Therapy," the teachings of which are incorporated by reference in their entirety.

A radioimmunoassay is schematically illustrated in Figure 2. (Also see Examples 1 and 3) For example, endogenous platelet activation can be assessed by an immunobinding assay comprising:

(a) obtaining a first and second sample comprising platelets, wherein each sample contains a preselected number of platelets;

(b) contacting said first sample with a platelet activation agonist, such as phorbol myristate acetate (PMA), ADP (adenosine diphosphate), thrombin, collagen, and/or TRAP (thrombin receptor activating peptide), under conditions suitable for activation of platelets in said first sample, preferably for a period of time effective to maximally activate said platelets, and preferably while maintaining the second

sample under conditions suitable for maintaining the endogenous platelet activation level;

(c) contacting said samples with a composition comprising an anti-P-selectin antibody, such as

(i) an anti-P-selectin antibody comprising a radioactive label; or

(ii) an anti-P-selectin antibody comprising a binding site for a second antibody which comprises a radioactive label,

preferably in an amount in excess of that required to bind the P-selectin expressed on the platelets, under conditions suitable for the formation of labeled complexes between said anti-P-selectin antibody and activated platelets; and

(d) determining (detecting or measuring) the formation of complex in said samples, wherein the amount of complex detected in said second sample as compared to that detected in said first sample is indicative of the extent of platelet activation in said second sample.

For example, a ratio reflecting the amount of complex detected in said second sample to that detected in said first sample can provide a measure of the extent of platelet activation in said second sample. Formation of complex can be assessed by determining the radioactivity present in the labeled complexes in each sample, wherein a ratio of the radioactivity of said second sample to said

first sample provides a measure of the extent of platelet activation in said second sample.

Preferably, the first and second samples are from the same donor. In particularly preferred embodiment, the first and second samples are collected at about the same time (e.g., obtained by dividing a sample from a donor, obtained from two samples collected in series).

The assay can also be performed on whole blood without a pre-isolation step or standardization of platelet number, thus substantially reducing processing time. For example, a sample of whole blood can be obtained from a donor whose level of platelet activation is to be determined and can be divided into two portions. One sample can be treated with a platelet agonist such as PMA to maximally activate platelets, while the other sample is not treated with activation agonists, but is maintained under conditions designed to maintain the endogenous (*in vivo*) activation level (e.g., by addition of activation inhibitors such as aprotinin, theophylline, apyrase and/or prostaglandin E_1). Radioactively labeled anti-P-selectin antibody is added to both samples and samples are maintained under conditions suitable for specific binding to P-selectin, and preferably until binding is complete. The extent of binding is the assessed. The samples can processed to separate complexes from unbound anti-P-selectin antibody. For example, samples can be diluted 1:6 with a buffer that does not alter platelet activation state, such as Tyrode's Modified Buffer, layered over a 30% sucrose barrier (e.g., in

preloaded microfuge tubes), and microfuged (e.g., for 4 minutes at 11,000 X g). The pellet with its bound radiolabeled anti-P-selectin antibody can be clipped and counted in a gamma counter. The percent of radioactivity in the endogenously activated sample compared with the maximally activated sample can be calculated and described as the Activation Index (AI) for the sample. In this manner, endogenous platelet activation can be measured as percent of total expressible P-selectin.

Another method of assaying levels of membrane bound P-selectin involves analysis with a volumetric capillary cytometry system. An example of a volumetric capillary cytometry system is IMAGN2000™ from Biometric Imaging, Mountain View, CA. As described in Figure 3 and Example 5, membrane bound P-selectin is measured using a P-selectin specific antibody or mixture thereof. Preferably, the antibody is labeled with a fluorophore. More preferably, the antibodies used are a mixture or cocktail of S-12 and W-40 each of which are labeled with fluorophore, Cy-5 (Amersham-Searle). The volumetric capillary cytometry system detects the number of events and the fluorescent intensity.

For example, the level of membrane bound P-selectin can be assessed by volumetric capillary cytometry system comprising:

(a) obtaining a sample comprising platelets,

(b) contacting said sample with a stabilizing reagent, such as Apyrase and Prostaglandin E1, to

prevent in vitro platelet activation and stabilize the P-selectin expressed on the platelets to obtain a measure of in vivo platelet activation,

(c) contacting or staining said samples with a composition comprising an anti-P-selectin antibody, such as an anti-P-selectin antibody comprising a fluorescent label, preferably in an amount in excess of that required to bind the P-selectin expressed on the platelets, under conditions suitable for the formation of labeled complexes between said anti-P-selectin antibody and activated platelets; and

(d) determining (detecting or measuring) the formation of complex in said samples, wherein the amount of complex detected indicated the level of membrane bound P-selectin in the sample.

Assessing the total platelet count aids in determining the extent of platelet activation and therefore, the total platelet count is preferably measured in addition to membrane bound P-selectin. The total platelet count is measured by contacting the sample with an antibody specific to essentially all platelets, and then detecting the number of events or fluorescence. Preferably, the antibody is an antibody specific for a receptor existing on essentially all platelets, such as glycoprotein GP IIb/IIIa, CD61, 10E5, CD41 and CD42. These antibodies are labeled with a fluorophore, namely Cy-5. (Amersham-Searle).

For example, a volumetric capillary cytometry system can assess the total platelet count in a method comprising:

(a) obtaining a sample comprising platelets,

(B) contacting or staining said samples with a composition comprising an anti-platelet antibody, such as an anti-GP IIB/IIIa antibody having a fluorescent label, preferably in an amount in excess of that required to bind the platelets, under conditions suitable for the formation of labeled complexes between said anti-platelet antibody and platelets; and

(d) determining (detecting or measuring) the formation of complex in said samples, wherein the amount of complex detected indicated the total platelet count in the sample.

Assaying for Detection of soluble P-selectin

Any method known now or developed later can be used for measuring soluble P-selectin. In a preferred embodiment, soluble P-selectin is determined using an ELISA assay or a sandwich ELISA assay. In a particularly preferred embodiment, the volumetric capillary cytometry system measures the level soluble P-selectin. Figure 4 illustrates one type of assay which a volumetric capillary cytometry system can perform (see also Example 6). In one embodiment, murine W40 is used as capture antibody and murine S12 is used as detector antibody.

For detection of soluble P-selectin in a suitable sample, a sample (e.g., blood) is collected, and preferably platelets are removed (partially or completely) from the sample, for example by preparation of serum or plasma (e.g., isolation of platelet poor plasma). Samples are preferably processed to remove platelets within a time suitable to reduce artificial increases in soluble P-selectin, such as those due to production of additional P-selectin (e.g., by secretion or proteolysis from platelets). For example, initiation of such processing within about one hour, and preferably immediately, is desirable. Samples can be further processed as appropriate (e.g., by dilution with assay buffer (e.g., ELISA diluent)). Additionally, the technician can add a reagent which stabilizes and prevents *in vitro* platelet activations. Examples of these stabilizing reagents are apyrase and PGE_1.

Thus, the present invention provides a method to determine the presence or absence of a thrombotic disorder by assessing the levels of membrane bound and soluble P-selectin. To determine a measurement for soluble P-selectin using an ELISA assay in a suitable sample such as serum, platelet poor plasma (PPP), the method comprises:

(a) combining

(i) a suitable sample,

(ii) a composition comprising an anti-P-selectin antibody as detector, such as

(a) biotinylated anti-P-selectin MAb (e.g., S12) and HRP-streptavidin, or

(b) HRP-conjugated anti-P-selectin Mab, and

(ii) a solid support, such as a microtiter plate, having an anti-P-selectin capture antibody bound (directly or indirectly) thereto,

wherein the detector antibody binds to a different P-selectin epitope from that recognized by the capture antibody, under conditions suitable for the formation of a complex between said anti-P-selectin antibodies and soluble P-selectin; and

(b) determining the formation of complex in said samples.

The solid support, such as a microtiter plate, dipstick, bead, or other suitable support, can be coated directly or indirectly with an anti-P-selectin antibody. For example, an anti-P-selectin antibody can coat a microtiter well, or a biotinylated anti-P-selectin Mab can be added to a streptavidin coated support. A variety of immobilizing or coating methods as well as a number of solid supports can be used, and can be selected according to the desired format.

In a particularly preferred embodiment, the sample (or soluble P-selectin standard) is combined with the solid support simultaneously with the detector antibody, and optionally with a one or more reagents by which detection

is monitored. For example, the sample such as PPP can be combined with the solid support simultaneously with (a) HRP-conjugated anti-P-selectin Mab, or (b) a biotinylated anti-P-selectin Mab and HRP-streptavidin.

A known amount of soluble P-selectin standard can be prepared and processed as described above for a suitable sample. This soluble P-selectin standard assists in quantifying the amount of P-selectin detected by comparing the level of P-selectin in the sample relative to that in the standard. In one embodiment, soluble truncated P-selectin is used as a standard.

A physician, technician, apparatus or a qualified person can compare the amount of detected complex with a suitable control to determine if the levels are elevated. For example, the level of soluble P-selectin following a vascular intervention procedure can be compared with a basal level for the individual such as a level determined prior to or at the time of the procedure, or with levels in normal individuals or suitable controls.

In one embodiment of the invention, the assay can be performed on serum isolated from whole blood of a donor which is allowed to clot in the absence of an anticoagulant with or without a clot-promoting gel. For example, a technician can collect whole blood in a vacutaininer without anticoagulant which may or may not have a clot-promoting gel plug that separates serum. After the blood clots, a technician can remove or harvest the serum from the top of the clotted cell pellet. The technician can

either assay the serum immediately thereafter in the ELISA format described above or freeze the serum at -70°C for later analysis. In the process of clotting, platelet microparticles are released which may be expressing P-selectin on their surface. Ultracentrifugation of serum at 107,000 X g for 3 hours showed that microparticle-bound P-selectin was not detected in the soluble P-selectin ELISA format described (Table 9). As seen in Table 9, the amount of soluble P-selectin detected in serum remained essentially unchanged after the sample was subjected to ultracentrifugation, a regimen which would remove microparticles from the serum. Therefore, the ELISA measures only soluble P-selectin in serum. In this assay, the mean amount of soluble P-selectin in serum is elevated over that observed in plasma (Table 9).

Table 9

Ultracentrifugation which removes microparticles does not significantly alter soluble P-selectin detected in the W40-S12 Mab ELISA:

Donor	Anticoagulant	Before 3 hour 107,000 X g centrifugation (soluble P-selectin ng/ml)	After 3 hour 107,000 X g centrifugation (soluble P-selectin ng/ml)
1	Serum with gel	138.2	132.1
1	Serum without gel	145.0	134.7
1	Plasma (ACD-A)	40.3	38.4
2	Serum with gel	134.0	139.2
2	Serum without gel	107.2	102.8
2	Plasma (ACD-A)	45.2	46.36

Table 9 shows that ultracentrifugation at 107,000 x g for 3 hours does not change the detection of P-selectin in the soluble P-selectin ELISA for plasma or serum. These results indicate that microparticles which would be removed by ultracentrifugation are not being detected in the soluble P-selectin ELISA, but that only plasma P-selectin is being detected. Abbreviation: ACD-A, Acid citrate dextrose, solution A.

Thus, in a preferred embodiment, the assay for measuring soluble P-selectin in a suitable sample comprises the following steps:

(a) obtaining a suitable sample such as plasma or serum;

(b) coating a microtiter plate with an anti-P-selectin capture antibody (e.g., W40) or adding a biotinylated anti-P-selectin capture antibody (e.g., W40) to a streptavidin coated solid support such as a microtiter plate;

(c) adding, preferably simultaneously, to said microtiter plate the sample to be tested (e.g., final dilution 1:4 with ELISA diluent) and a composition comprising a detector antibody and optionally a reagent for detection, such as

(i) HRP-conjugated anti-P-selectin detector antibody (e.g., HRP-S12), or

(ii) a composition comprising biotinylated anti-P-selectin detector antibody (e.g., biotinylated Mab S12) and HRP-streptavidin,

wherein the anti-P-selectin detector antibody binds to a different P-selectin epitope from that bound by the capture antibody, and incubating same under conditions suitable for the formation of a complex between said anti-P-selectin antibodies and soluble P-selectin, preferably under conditions which maximize binding;

(d) separating complexes comprising capture antibody, soluble P-selectin and detector antibody (e.g., by washing); and

(e) determining the amount of soluble P-selectin in said complexes.

Typical assays for P-selectin are sequential assays in which a plate is coated with first antibody, plasma is added, the plate is washed, second tagged antibody is added, and the plate is washed and bound second antibody is quantified. However, binding kinetics revealed that in a simultaneous format, the off-rate of the second antibody was decreased and the assay was more sensitive. Thus, a simultaneous format in which the solid support is coated with a capture antibody (e.g., W40), and plasma and detector antibody (e.g., S12) are added simultaneously, can achieve enhanced sensitivity and is preferred.

A variety of methods can determine the amount of soluble P-selectin in complexes. For example, when HRP is used as a label, a suitable substrate such as OPD can be added to produce color intensity directly proportional to the bound anti-P-selectin Mab (assessed e.g., by optical density), and therefore to the soluble P-selectin in the sample.

A technician, physician, qualified person or apparatus can compare the results to a suitable control such as a standard, levels of P-selectin in normal individuals, and baseline levels of P-selectin in a sample from the same donor. For example, the assay can be

performed using a known amount of soluble P-selectin standard in lieu of a sample, and a standard curved established. One can relatively compare known amounts of the soluble P-selectin standard to the amount of complex formed or detected.

The volumetric capillary cytometry system also measures soluble P-selectin. (Illustrated in Figure 3). The antibody detection concepts used in the ELISA and sandwich ELISA as described above apply to measurements obtained from using the volumetric capillary cytometry system. The above ELISA methods described can be adapted so that the support surface and method of detection utilized is suitable for measurement with a volumetric capillary cytometry system.

As described above, a technician obtains a suitable sample. Samples are preferably processed to remove platelets within a suitable time, preferably within one hour, to reduce artifactual increases in soluble P-selectin, such as those due to production of additional P-selectin. Additionally, the technician can add a reagent which stabilizes and prevents *in vitro* platelet activations. Examples of these stabilizing reagents are apyrase and PGE_1. An antibody specific to P-selectin is coated or immobilized on a support surface, such as a bead, solid support strip, or modified capillary surface. The sample is contacted with the coated surface. The coated antibody is preferably W40. This coated antibody may be detectably labeled. In the preferred embodiment, a

fluorofore such as Cy5 labels the coated antibody. Alternatively and more preferably, another antibody specific to P-selectin or a complex between P-selectin and the coated antibody can contact the sample. This second antibody is detectably labeled with a fluorofore such as Cy5. The volumetric capillary cytometry system can then determine the fluorescent intensity as a measure of soluble P-selectin.

Thus, in a particularly preferred embodiment, the assay used in conjunction with the volumetric capillary cytometry system with for measuring soluble P-selectin in a suitable sample comprises the following steps:

(a) obtaining a suitable sample, for example plasma;

(b) coating a support surface with an anti-P-selectin capture antibody (e.g., W40) or adding a biotinylated anti-P-selectin capture antibody (e.g., W40) to a streptavidin coated solid support;

(c) adding, preferably simultaneously, the sample to be tested and a composition comprising a detector antibody and a reagent for detection, such as a fluorophore (e.g., Cy5-S12) wherein the anti-P-selectin detector antibody binds to a different P-selectin epitope from that bound by the capture antibody, and incubating same under conditions suitable for the formation of a complex between said anti-P-selectin antibodies and soluble P-

selectin, preferably under conditions which maximize binding; and

(D) determining the amount of soluble P-selectin in said complexes using a volumetric capillary cytometry system or a similar apparatus.

The P-selectin profile as a measurement of platelet activation comprises the individual determinations of the level of platelet membrane bound P-selectin and the level of soluble P-selectin in a sample. Accordingly, the individual results of the methods discussed in this document can be combined to determine the P-selectin profile. The same kit or apparatus may utilize these methods to determine the measurement of the P-selectin profile.

Exemplification:

The following Examples illustrate the claimed invention and are not intended to be limiting in any way:

Example 1. Radioimmunoassay (RIA) for the detection of platelet bound P-selectin and platelet activation

The radioimmunoassay method used in these *in vitro* and *in vivo* studies is described schematically in Figure 2. As shown therein, the method can be used to determine the level of platelet activation by measuring the expression of platelet-bound P-selectin. All *in vitro* and *in vivo* determinations of platelet bound P-selectin described in

the examples were performed according to the following protocol.

For *in vitro* studies and some *in vivo* studies, whole blood (8.5 cc) was collected by venipuncture using a 19-gauge needle in two 10-ml vacutainer tubes containing ACD-A (1.5 cc) as anticoagulant. Where the patient had an arterial catheter in place, blood was collected from the in-dwelling arterial line into two plastic syringes containing 1.5 cc ACD-A anticoagulant. In this latter case, each syringe was filled to the 10 cc mark (8.5 cc draw).

The blood with anticoagulant from one vacutainer or syringe was immediately transferred into a polypropylene centrifuge tube (15 ml) containing one premeasured aliquot of apyrase (final concentration 1 U/mL)(Sigma, St. Louis, MO, Catalog No. A 9149) and prostaglandin E_1 (PGE_1, final concentration 1 μM)(Sigma, St. Louis, MO, Catalog No. P 5515). Apyrase and PGE_1 prevent *in vitro* platelet activation and stabilize the P-selectin expressed on platelets so that the P-selectin expressed on platelets in this blood sample represents the actual *in vivo* level of platelet activation. Blood from the second vacutainer or syringe was immediately transferred into an empty polypropylene centrifuge tube (15 ml) and was subsequently treated with a platelet agonist to establish maximal P-selectin expression for the donor. Platelet rich plasma (PRP) was prepared from whole blood by centrifugation of both polypropylene tubes for 6 minutes at 600 X g. The

yellow supernatant PRP was removed from each of the tubes (with or without apyrase and PGE_1) with plastic pipettes and placed into empty polypropylene tubes.

Platelet poor plasma (PPP) was prepared by centrifuging (10 minutes at 1900 x g) the red cell pellet remaining in the polypropylene centrifuge tube after the preparation of PRP. Platelet counts in PRP were determined using a Coulter counter and the final platelet concentration was adjusted to 1.0×10^8 platelets/mL using the appropriate PPP (i.e., with apyrase and PGE_1 or without apyrase and PGE_1).

Platelet bound P-selectin expression was measured in a radioimmunoassay (RIA) using an ^{125}I-labeled murine anti-human P-selectin monoclonal antibody (MAb) designated S12. The S12 monoclonal antibody, which is specific for P-selectin, reacts minimally with unstimulated human platelets, but binds extensively to platelets after activation with thrombin (McEver, R.P and M.N. Martin, "A Monoclonal Antibody to a Membrane Glycoprotein Binds Only to Activated Platelets", *J. Biol. Chem., 259 (15)*: 9799-9804 (1984), the teachings of which are incorporated herein by reference in their entirety). In the RIA, the binding of ^{125}I-labeled anti-P-selectin monoclonal antibody S12 to: (1) P-selectin molecules expressed on the surface of unstimulated platelets (treated with apyrase and PGE_1 to maintain *in vivo* P-selectin expression), and (2) P-selectin molecules expressed on the platelets of the same donors after stimulation with a final concentration of 0.5 μM

phorbol myristate acetate (PMA)(Sigma, St. Louis, MO, Catalog No. P 8139) (no apyrase or PGE_1), which causes maximal P-selectin expression on platelets, was determined.

To perform the RIA, Mab S12 was radioiodinated as described previously (Wagner, C. *et al.*, *Blood, 88*: 907 (1996), the teachings of which are incorporated herein by reference in their entirety). One 0.5 ml aliquot of PRP adjusted to 1.0 x 10^8 platelets/ml containing apyrase and PGE_1 was transferred to a polypropylene microfuge tube (500 µl capacity) containing 20 µl of Modified Tyrodes Buffer (MTB), while a second 0.5 ml aliquot of PRP (without apyrase or PGE_1) was transferred to a similar microfuge tube containing 20 µl of PMA (final concentration in the PRP of 0.5 µM). Both tubes were gently inverted and incubated for 15 minutes at room temperature.

^{125}I-labeled anti-P-selectin Mab S12 (final concentration 2 µg/ml in the PRP) was added to each microfuge tube, and the tubes were incubated for 30 minutes at room temperature. Specific activity was typically in the range of 2 to 4 µCi/µg. Aliquots (100 µl) of PRP were removed from each microfuge tube and layered over 30% sucrose (200 µl)(J.T. Baker, Phillipsburg, NJ, Catalog No. 4097-04) preloaded in slender (400 µl) polypropylene microfuge tubes. Samples were microfuged for 4 minutes at 11,000 x g causing the platelets with their bound ^{125}I-S12 to pellet, and to be separated from the free ^{125}I-S12 by the sucrose barrier. The platelet pellet was separated from the supernatant, containing free ^{125}I-labeled anti-P-

selectin Mab S12, by clipping off the bottom of the microfuge tube and determining the bound counts per minute (cpm) on a gamma counter.

An Activation Index (AI) was calculated for each donor/patient. The activation index is the percent of total P-selectin (determined in the PMA activated sample) which is expressed by the platelets in the *ex vivo* sample (endogenous platelet activation).

$$\text{Activation Index} = \frac{\text{cpm in pellet of } \textit{ex vivo} \text{ PRP}}{\text{cpm in pellet of PMA activated PRP}} \times 100$$

The activation index (AI) calculated for eight (n = 8) normal donors was 2.7 ± 1.5.

Example 2. Measurement of soluble P-selectin by ELISA

The ELISA method used in these *in vitro* and *in vivo* studies is described above. As shown herein, the method can be used to determine the level of platelet activation by determining the amount of soluble P-selectin in a sample. All *in vitro* and *in vivo* determinations of soluble P-selectin described in the examples were performed according to the ELISA protocol described below.

Selection of Antibodies for ELISA

To select monoclonal antibodies for a sandwich ELISA, the antigen binding kinetics of three anti-P-selectin murine monoclonal antibodies (W40, S12, and G1) were

examined on the BIAcore™ instrument (Pharmacia Biosensor, Uppsala Sweden), a surface plasmon resonance detection system which is applied to kinetic, binding site and concentration analysis. Each monoclonal antibody was captured on a BIAcore™ chip with a rabbit anti-mouse Fc specific antibody. Soluble P-selectin was passed over the chip, and increasing mass (indicative of the antibody on-rate) was measured. After antigen saturation had been attained, buffer was passed over the chip and antigen off-rate was seen as decreasing mass. Association rates for each of the monoclonal anti-P-selectin antibodies were equivalent, while off-rates differed significantly. Both S12 and G1 antibodies immediately exhibited fast dissociation of antigen (off-rate). In contrast, W40 did not show a loss of P-selectin when flow of antigen was replaced with buffer alone. Thus, the BIAcore™ results demonstrated a much slower off-rate of soluble P-selectin from W40 than from either S12 or G1 monoclonal antibodies.

BIAcore™ experiments also revealed that the off-rate of soluble P-selectin from S12 was unexpectedly altered when P-selectin was bound with W40 antibody. When S12 was coated on a BIAcore™ chip and soluble P-selectin was bound to saturation, soluble P-selectin was immediately released when antigen flow was discontinued. However, when W40 was used to capture soluble P-selectin on the chip and S12 was allowed to bind to the captured soluble P-selectin, S12 remained attached when buffer was passed over the chip.

The off-rate of S12 from soluble P-selectin was decreased when P-selectin was captured by W40.

Accordingly, a simultaneous format using W40 as the capture antibody and S12 as the detection antibody was selected to maximize sensitivity.

The Materials used in this assay are as follows:

Reagents/Supplies	Supplier/Manufacturer	Catalog #
Nunc MaxiSorp™ 96-well microtiter plates	VWR	62409-004
PBS 10X stock	JRH Bioscience	59331-79P
Bovine Serum Albumin (BSA)	Intergen	
Polyoxyethylene sorbitan monolaurate (Tween 20)	Sigma	P7949
Soluble P-Selectin, truncated P-selectin (tPS)	Centocor Inc.	
Horseradish Peroxidase-conjugated Streptavidin (SA-HRP)	Jackson Immunoresearch Labs	016-030-084
Murine mAb W40 IgG	Centocor Inc.	
Biotinylated murine mAb S12 IgG	Centocor Inc.	
Citric Acid	J.T. Baker	0118-01
Sodium Phosphate Dibasic	Sigma	S9763
30% H_2O_2	Sigma	H1009
O-phenylenediamine dihydrochloride (OPD)	Sigma	8287
B2TT antibody	Centocor Inc.	
4N Sulfuric acid, H_2SO_4 prepared from concentrated acid	J.T. Baker	968102

The following buffers were prepared prior to performing the assay:

1X PBS	Dilute 10X PBS 1:10 with deionized H_2O
PBS / 1% BSA	Dissolve 5 grams BSA in 500 ml PBS and filter (0.2 μm)
PBS / 1% BSA / 0.05% Tween 20 / 25 μg/ml B2TT (azide free)	Dissolve 5 grams BSA in 500 ml PBS; add 0.250 ml Tween 20; Add 1.25 ml B2TT @ 10 mg/ml; and filter (0.2 μm)
PBS / 0.05% Tween 20	Add 0.5 ml Tween 20 per liter of PBS and mix thoroughly
Citrate/Phosphate Buffer (1 liter)	4.2 g Citric Acid (20 mM); 7.1 g Sodium phosphate dibasic (anhydrous) (50 mM); Add 900 mls water and adjust pH to 5.0; QS to 1.0 liter with water and filter (0.2 μm).
OPD substrate solution (25 mls)	Dissolve three 10 mg OPD tablets in 25 mls citrate/phosphate buffer and add 40 μl 30% H_2O_2. Prepare just before use.
4N Sulfuric Acid	Add 20 mls concentrated sulphuric acid to 160 mls deionized H_2O.

Murine W40 IgG_1 Purification

Murine W40 IgG_1, a murine monoclonal antibody specific for human P-selectin (Johnston, G.I. *et al., J. Biol. Chem., 264*: 1816-1823 (1989), the teachings of which are incorporated herein by reference in their entirety), was prepared as ascites fluid and was purified by "high salt" protein A chromatography. Ascites fluid was thawed from -70°C and filtered using several glass prefilters and 0.2 μm membrane syringe filters. The ascites fluid was then adjusted to 3M NaCl with granular sodium chloride and the pH increased to 8.9 by addition of 1M glycine pH 9.6. Protein A Hi-trap columns were equilibrated on a Pharmacia FPLC in MAPS buffer (3M NaCl, 1.5 M glycine, pH 8.9). The ascites fluid, adjusted for salt and pH, was loaded on the Protein A column and flow-through was collected when the OD_{280} rose above baseline. Once sample loading was complete, the column was washed with additional MAPS buffer until the OD_{280} returned to baseline. Bound antibody was first eluted with 0.1M citrate pH 5.5. Collection of eluate was begun and stopped as the OD_{280} rose above and returned to baseline. The pool of eluted antibody was immediately neutralized with the addition of 1/3 final volume 1M Tris, pH 8.0. Other non-W40 IgG proteins bound to the column were removed by washing with 0.1 M citrate pH 3.5. This eluate was also collected and neutralized as described above.

The pH 5.5 eluate was then concentrated using centriplus™ concentrators and dialyzed into PBS using a

Slide-A-lyzer™ apparatus (Pierce). Finally the sample was 0.2 μm filtered and the concentration determined by OD_{280}.

Murine S12 IgG_1 Biotinylation

Murine S12 IgG_1 antibody was purified from hybridoma tissue culture supernatant using Protein A Sepharose column chromatography, and was dialyzed into 200 mM $NaHCO_3$, 150 mM KCl, pH 8.5 and concentrated to 3.95 mg/ml for biotinylation. Biotinylation was carried out with a 30:1 molar excess of NHS-LC-biotin (Pierce) to murine S12 IgG. Briefly, mS12 IgG was transferred to a 5 ml polypropylene tube; NHS-LC-biotin was weighed out and quickly reconstituted to 4 mg/ml in DI water. The appropriate amount of NHS-LC-biotin was transferred to the reaction tube containing S12 IgG and mixed at room temperature for 1 hour.

Free biotin was removed from the biotinylated murine S12 IgG antibody by transferring to a Slide-A-lyzer™ for dialysis into PBS. Finally, the antibody was 0.2μm filtered and the concentration determined by OD_{280}.

Truncated P-selectin Generation and Purification

Transfected 293 Tissue Culture Methods

Human 293 kidney cells (ATCC CRL 1573) were obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, and were transfected with a construct which directs the expression of soluble truncated P-selectin (tPS) from pRC/RSV (Invitrogen)(Ushiyama, S. *et*

al., J. Biol. Chem., 268: 15229 (1993), the teachings of which are incorporated herein by reference in their entirety). Transfectants were cultured in αMEM containing 10% FBS and supplemented with L-glutamine, sodium pyruvate, NEAA and geneticin (G-418) in T-150 flasks. When cells reached confluency, supernatants were decanted, centrifuged to remove cells and debris, and stored at 4°C for purification.

Truncated P-selectin Affinity Purification

Tissue culture supernatant from 293 cells containing truncated P-selectin (tPS) was collected and pooled for processing. A 25 ml murine G1 affinity column was prepared using the anti-P-selectin murine monoclonal antibody G1 (Geng, J.-G. *et al., Nature, 343*: 757-760 (1990)), and the column was equilibrated with 5 column volumes of 20 mM Tris, 100 mM NaCl, pH 8.3 at 4°C. Tissue culture supernatant was loaded onto the column and flow-through collected. When sample loading was complete, the column was washed with equilibration buffer until the OD_{280} returned to baseline. The affinity column was then washed with 5 column volumes of 20 mM Tris, 1M NaCl, pH 8.3. The column was again equilibrated with 5 column volumes 20 mM Tris, 100 mM NaCl, pH 8.3. Bound tPS was eluted with nine column volumes of 100 mM sodium acetate, 100 mM NaCl, pH 4.1. The column was re-equilibrated in 20 mM Tris, 100 mM NaCl, pH 8.3 containing 0.1% NaN_3 and stored for future use.

Eluate fractions were immediately neutralized with 1M MOPS pH 7.9 and concentrated using a Centriplus™ concentrator. Purified tPS was then buffer exchanged into 20 mM MOPS, 100 mM NaCl, pH 7.5 using a Slide-A-lyzer™ apparatus. Finally, the sample was 0.2 μm filtered and concentration determined by OD_{280} extinction coefficient 12.3.

Sandwich ELISA for Detecting Soluble P-Selectin

The sandwich ELISA method for assaying soluble P-selectin levels used the following procedure. Table 10 illustrates the final concentrations of reagents utilized in the sandwich ELISA.

Table 10. Final concentrations of reagents used in the ELISA.

Component	Prepare	Final Conc.	Volume added to dilution plate
A1. Sample	1:2 dilution	1:4 dilution	100 µl
A2. tPS Standards*	640 ng/ml - 6.4 ng/ml	320 ng/ml - 3.2 ng/ml	100 µl
B. Streptavidin-HRP	(1:25,000)	1:100,000	50 µl
C. Biotinylated S12 IgG	5.0 µg/ml	1.25 µg/ml	50 µl

* A six point standard curve was prepared by serially diluting tPS from 320 ng/ml to 3.2 ng/ml. Serial dilutions were carried out by transferring 66 µl standard into wells containing 100 µl of buffer, mixing and transferring again.

Whole blood (8.5 cc) was collected by venipuncture using a 19-gauge needle in two 10-ml vacutainer tubes containing ACD-A (1.5 cc), heparin or EDTA as anticoagulant. Where the patient had an arterial catheter in place, blood was collected from the in-dwelling arterial line into a plastic syringe containing 1.5 cc ACD-A, heparin or EDTA as anticoagulant. The syringe was filled to the 10 cc mark (8.5 cc draw).

The blood with anticoagulant from the vacutainer or syringe was immediately transferred to a polypropylene centrifuge tube. Platelet poor plasma (PPP) was produced by centrifuging the whole blood for 20 minutes at 1900 x g. The PPP was removed from the cell pellet by plastic transfer pipet and was assayed in the ELISA format described below or was aliquoted and frozen at -70°C for later analysis.

Soluble P-selectin was measured in an enzyme-linked immunosorbent assay (ELISA) by coating 96-well MaxiSorp™ (Nunc) microtiter plates with murine anti-P-selectin Mab W40 IgG, by adding 100 μl of antibody (at a concentration of 5 μg/ml in PBS) to each well. Plates were incubated at 4°C for approximately 18 hours. The coated microtiter plates were washed three times with 200 μl/well of PBS and blocked by the addition of 200 μl/well of PBS containing 1% bovine serum albumin (BSA, Fraction V, Sigma, St. Louis, MO) for 1 hour at 37 °C. Blocked plates were incubated for 2 hours at 37 °C with the following simultaneously added components: soluble P-selectin standards or donor plasma

samples, horseradish peroxidase-conjugated streptavidin and biotin conjugated-anti-P selectin antibody S12 IgG, which binds to a P-selectin epitope which is distinct from that recognized by W40 IgG. PBS containing 1% BSA, 0.05% Tween 20, and 25 μg/ml B2TT (mouse Ig to eliminate non-P-selectin specific human anti-mouse reactivity; Centocor, Malvern, PA) was used as the diluent for all assay components. Plasma samples were evaluated at a final concentration of 1:4 in the assay diluent. After incubation of the samples and standards, plates were washed four times with 200 μl/well of PBS with 0.05% Tween 20. Color was developed by the addition of 100 μl/well of the HRP substrate O-phenylenediamine dihydrochloride (OPD). Color development was stopped after 20 minutes by the addition of 100 μl/well of 4N H_2SO_4.

Plates were read at 490 nM on a Molecular Devices plate reader. Softmax™ software was used to analyze the data. A standard curve was generated by plotting the mean absorbance for known quantities of soluble P-selectin produced by a human kidney cell line (293 cells) transfected with a gene producing a truncated form of P-selectin which does not include the transmembrane portion of the molecule (Ushiyama, S. *et al.*, *J. Biol. Chem.*, *268*: 15229 (1993), the teachings of which are incorporated herein by reference in their entirety). Figure 5 shows a typical standard curve derived from performance of a soluble P-selectin ELISA for concentrations of soluble P-selectin from 3.2 to 320 ng/ml. As seen in Figure 5, the

mean absorbance for each standard value was plotted on the Y-axis and the concentration of P-selectin on the X-axis. The points were fitted using a log-log curve fitting program. The concentration of soluble P-selectin in samples was determined from the standard curve multiplied by the appropriate dilution factor.

Normal levels of endogenous soluble P-selectin were assessed in volunteer donors (n = 12) who were not suffering from coronary artery disease. Table 11 shows the normal ranges of soluble P-selectin in serum collected in vacutainers with or without clot-promoting gel and in plasma drawn into the following anticoagulants: ACD-A, heparin, and EDTA.

Table 11. Soluble P-selectin Levels in Normal (n = 12) plasma and serum

Anticoagulant	Plasma P-selectin (ng/mL)	Serum P-selectin (ng/mL)
ACD	25.6 ± 7.5	
Heparin	32.9 ± 7.7	
EDTA	31.3 ± 6.8	
No Gel		73.8 ± 23.6
Gel		86.12 ± 23.6

Table 11 gives the mean ± standard deviation of soluble P-selectin found in normal donors (n = 12) when the soluble P-selectin ELISA is performed on plasma isolated from whole blood collected into the anticoagulants, ACD-A, heparin, or EDTA or on serum collected with or without clot-promoting gel. Soluble P-selectin is considered to be significantly elevated if its value is 3 standard deviations above the normal mean for that particular type of plasma or serum sample.

Performance Characteristics

The intra-assay variability (precision within an assay) for the soluble P-selectin ELISA format was determined by adding known amounts of soluble P-selectin to human plasma which had been collected in ACD-A anti-coagulant. In particular, four plasma samples were spiked with high (600 ng/ml), medium (300 ng/ml), low (40 ng/ml) or no (0 ng/ml) tPS. (The endogenous level of soluble P-selectin in the plasma was considered zero added P-selectin for purposes of this assay.) Twenty-one replicates for each value were determined on the same microtiter plate to derive the intra-assay variability (i.e., each of the four samples was assayed on one plate in replicates of 21).

As shown in Table 12, low, medium, and high amounts of P-selectin were determined in the assay. As indicated in Table 12, a coefficient of variation (CV) less the 10% was achieved for all soluble-selectin levels.

Table 12. Intra-assay variability of the soluble P-selectin ELISA for human plasma

Sample	Zero 0 ng/ml spike	Low 40 ng/ml spike	Medium 300 ng/ml spike	High 600 ng/ml spike
n	21	21	21	21
Mean (ng/ml)	14.0	48.0	315.7	590.8
Std. Dev.	0.54	3.75	11.40	28.17
CV (%)	3.8	7.8	3.6	4.7

The inter-assay variability (precision between assays) of the soluble P-selectin ELISA format was determined in ten (10) different assays in which six replicate determinations of four plasma samples (ACD-A as anticoagulant) were spiked with zero (endogenous soluble P-selectin only), low (20 ng/ml), medium (250 ng/ml), and high (600 ng/ml) amounts of soluble P-selectin (tPS). As can be observed, all CV's were ≤ 15% (Table 13).

Table 13. Inter-assay variability of the soluble P-selectin ELISA for human plasma

Sample	Zero 0 ng/ml spike	Low 20 ng/ml spike	Medium 250 ng/ml spike	High 600 ng/ml spike
n	10	10	10	10
Mean (ng/ml)	26.4	40.1	246.7	545.5
Std. Dev.	2.90	2.37	11.21	29.7
CV (%)	11.0	5.9	4.5	5.5

Example 3. Use of radioimmunoassay (RIA) to measure induction of expression of platelet P-selectin in response to PMA

Platelets in plasma from a healthy donor were isolated from whole blood as described in Example 1 and were activated by the platelet activation agonist PMA at various final concentrations ranging from 5 to 500 nM. P-selectin in the activated platelets was translocated to the membrane in response to PMA in a dose-dependent manner which was measured by the binding of the iodinated anti-P-selectin antibody, ^{125}I-S12. Greater amounts of antibody represented by higher counts per minute were bound with increasing concentrations of PMA. Figure 6A and Figure 6B show the results of this titration. In Figure 6A, the data are presented as counts bound and in Figure 6B, the Activation Index (AI) for each titration of PMA is calculated according to the formula set forth in Example 1.

Example 4: The use of Flow Cytometry to measure membrane bound P-selectin.

Flow cytometry is one method to determine the level of platelet P-selectin and its result contributes to the measurement of the P-selectin profile.

Subpart a) Preparation of Platelet Rich Plasma (PRP) from whole blood for the performance of Flow Cytometry:

Flow cytometry is a method for determining the platelet P-selectin in patient samples, as discussed in

this document. The soluble P-selectin was determined using an enzyme-linked immunosorbent assay (ELISA) protocol. Normal values for platelet activation as measured by membrane bound P-selectin and normal levels of circulating soluble P-selectin were determined for apparently healthy volunteer donors.

For *in vitro* studies, whole blood was collected by venipuncture using a 19-gauge needle into a vacutainer tube containing either ACD solution A (Becton Dickinson, Catalog No 364606) or ACD solution B (Becton-Dickinson, Catalog No 364816) as anticoagulant. Within 30 minutes of the draw, the blood with anticoagulant from one vacutainer was transferred into a 15 mL polypropylene centrifuge tube (VWR, Catalog No 21008-102) containing one premeasured aliquot of apyrase (final concentration 1 U/mL (Sigma, St. Louis, MO, Catalog No. A 9149) and prostaglandin E_1 (PGE_1, final concentration 1 μM) (Sigma, St. Louis, MO, Catalog No P 5515) in Modified Tyrodes Buffer (MTB) (20mM HEPES, 187 mM NaCl, 4mM KCl, 50 mM Na_2HPO_4, 1mM $MgCl \cdot 6H_2O$, 5.5mM glucose, 1% bovine albumin). The use of polypropylene and the addition of apyrase and PGE_1 prevent *in vitro* platelet activation and stabilize the P-selectin expressed on platelets so that the P-selectin expressed on platelets in the blood sample represents the actual *in vivo* level of platelet activation. Gentle mixing and handling of the samples and the performance of all procedures at room temperature are also important in preventing *in vitro* activation. Platelet rich plasma is prepared by

centrifuging the whole blood at 600 x g in a Beckman GS-6KR centrifuge or equivalent, equipped with a rotor with swinging bucket, with no brake for 3 minutes (blood volumes of 3-6 mL) or 6 minutes (blood volumes of 10mL) at room temperature. The supernatant platelet rich plasma was removed from each centrifuge tube using a plastic transfer pipette (Sarstedt, No 86.1174 or equivalent) and transferred to a 5 mL polypropylene snap cap tube (VWR, Catalog No 60819-728 or equivalent) and capped to minimize CO_2 release.

Subpart b) Processing the Platelet Rich Plasma for flow cytometric analysis: Platelets in platelet rich plasma are stained with P-selectin specific monoclonal antibodies for flow cytometric analysis. Normal donors have a low percent of activated platelets or platelets which are expressing P-selectin. These normal donors provided samples which were used to determine the level of significant platelet activation. Patient samples show significant platelet activation when the percent of total platelets which are positive for P-selectin is greater than or equal to 2 standard deviations above the mean for the percent positive platelets observed in apparently healthy volunteer donors.

Platelet rich plasma for flow cytometric analysis is diluted 1:6 in Modified Tyrodes Buffer(MTB) and inverted to mix gently. Three stained samples are prepared by aliquoting 45 μL of diluted platelet rich plasma into each of two tubes containing 5 μL of Modified Tyrodes Buffer and

one tube containing 5 μL of phorbol 12-myristate 13-acetate (PMA) (Sigma, P-8139 or equivalent) to produce a final concentration of 20 nM PMA. The 20 nM PMA maximally activates the platelet rich plasma during a 15 minute incubation at room temperature and this sample acts as a control to show that the P-selectin specific antibody binds to its ligand in this system. 30 μL of mouse IgG FITC (50 μg/mL, Becton Dickinson, Catalog No. 349041 or equivalent) is added to one of the unactivated tubes containing PRP and buffer. This sample is the negative isotype matched control tube. 30 μL of the fluorescein-conjugated P-selectin specific antibody S12-FITC is added to one non-activated sample (test sample) and to the maximally activated (positive control) samples of diluted PRP. After a 20 minute incubation at room temperature, samples were fixed by the addition of 80 μL of 2% paraformaldehyde (Electron Microscopy Sciences Catalog No 15712-S or equivalent) for 30 minutes at room temperature. Samples were stored at 2-8°C for up to 72 hours prior to flow cytometric analysis.

Subpart c) Flow Cytometric analysis of platelets in PRP: The prepared samples were analyzed for platelet P-selectin expression using a FACSan flow cytometer (Becton Dickinson, San Jose, CA). The instrument is equipped with a 15-mW argon-ion laser at a wavelength of 488nm. The FITC fluorescence is detected using a 530-nm band pass filter. Platelets were identified by their forward and side scatter on a log scale. The characteristic platelet light scatter

was confirmed using a 10E5-FITC antibody to stain the GP IIB/IIIa receptor found on all platelets. A collection gate was drawn around the platelet population and used to collect 10,000 platelets at a rate of 400-1000 events per second. Analysis of the platelet region was performed using CellQuest 40. In the analysis method used, the log FL1 histograms of the control mouse IgG-FITC and the S12-FITC are overlaid. A statistical marker is positioned to result in 1% of the cells stained with the mouse control being considered positive. Keeping the marker in the same position, the percent of S-12-FITC stained cells which are positive for P-selectin expression is determined. The percent positive cells in the maximally activated samples is also assessed to insure that the S12-FITC antibody is binding optimally to P-selectin.

The inventors generated a series of color histograms illustrating the diagnostic sensitivity from the flow cytometry assay shown in Figure 1. The histograms were not included in the application because they are in color. The purpose of the histogram was to show the linearity of the addition of an increasing percent of fully activated platelets to whole blood containing non-activated platelets. Blood was drawn from a donor and divided into two parts one of which was not activated and the other was activated with PMA. Activated platelets were added to the non-activated sample to increasing percent of the total. The effect of the addition of activated platelets was determined by flow cytometric measurement of the resulting

percent positive platelets. In this particular experiment, the basal activation of the non-activated sample is 5.06 %. The addition to the non-activated blood sample of maximally activated platelets amounting to 1% of the total number resulted in the detection of 6.41 % activated platelets. The addition of 5% activated platelets to the base of 5.06% resulted in 9.39% activation being determined by this method. This experiment shows that the percent of activated platelets present in a sample of whole blood can be accurately assessed with the sensitivity required to make this a viable method for activated platelet determinations.

Example 5: The use of a Volumetric Capillary Cytometry System for measuring membrane bound P-selectin:

The volumetric capillary cytometry system utilized to measure membrane bound P-selectin was the IMAGN2000™ from Biometric Imaging, Mountain View, CA.

Subpart a) Obtaining and preparing a suitable sample for measuring membrane bound P-selectin using a volumetric capillary cytometry system:

Whole blood was collected by venipuncture using a 19-gauge needle into a vacutainer tube containing either ACD solution A (Becton Dickinson, Catalog No 364606) or ACD solution B (Becton-Dickinson, Catalog No 364816) as anticoagulant. Within 30 minutes of the draw, the blood with anticoagulant from one vacutainer was transferred into a 15 mL polypropylene centrifuge tube (VWR, Catalog No

21008-102) containing one premeasured aliquot of apyrase (final concentration 1 U/mL (Sigma, St. Louis, MO, Catalog No. A 9149) and prostaglandin E_1 (PGE_1, final concentration 1 μM) (Sigma, St. Louis, MO, Catalog No P 5515) in Modified Tyrodes Buffer (MTB) (20mM HEPES, 187 mM NaCl, 4mM KCl, 50 mM Na_2HPO_4, 1mM $MgCl \cdot 6H_2O$, 5.5mM glucose, 1% bovine albumin). The use of polypropylene and the addition of apyrase and PGE_1 prevent *in vitro* platelet activation and stabilize the P-selectin expressed on platelets so that the P-selectin expressed on platelets in the blood sample represents the actual *in vivo* level of platelet activation. Gentle mixing and handling of the samples and the performance of all procedures at room temperature are also important in preventing *in vitro* activation.

Subpart b) Staining a suitable sample for use with volumetric capillary cytometry system:

Whole blood containing apyrase and PGE_1 was stained with a cocktail of the P-selectin specific antibodies S12 and W40 which had been labeled with the fluorophore Cy-5 (Amersham-Searle). Cy5-labeled S12/W40 cocktail 5μg/mL (10X concentration) in Modified Tyrodes Buffer was kept frozen at -20°C in 50μL aliquots. A fresh aliquot was thawed as needed and discarded. To stain platelets for P-selectin, 45 μL of whole blood was aliquoted into an amber tube (Sarstedt Catalog No 72.694.034 or equivalent) containing 5μL of the S12-Cy5/W40-Cy5 cocktail at a final concentration of each of 0.5μg/mL and incubated at room temperature for 20 minutes. At the end of the staining

incubation, platelets were diluted and fixed by the addition of 1200 μL of 2% paraformaldehyde (Electron Microscopy Sciences Catalog No 15712-S or equivalent).

The stained, fixed, and diluted whole blood sample (40 μL) was placed in the well of a plastic capillary (Catalog No VC120, Biometric Imaging, Mountain View, CA) and the fluorescence intensity and number of events within the platelet size gate was determined in the IMAGN2000 instrument (Biometric Imaging, Mountain View, CA).

Subpart c) The total platelet count using the volumetric capillary cytometry system:

The total platelet count in each sample was determined on the IMAGN2000 Biometric Imaging instrument using a Cy-5 labeled CD61 antibody (Becton Dickinson) or a 10E5-Cy5 antibody (Centocor Inc., Malvern PA) both of which bind to essentially all platelets. Cy5-labeled CD61 and 10E5 at 5μg/mL (10 X) were stored frozen (-20°C) in 200 μL aliquots. During use, the reagent is stored at 4°C. Unused refrigerated reagent is discarded monthly. The total platelet count was performed in whole blood by transferring 5 μL of blood to a 12 x 75 mm polypropylene tube (Falcon 2063 or equivalent) containing 5 mL of Modified Tyrode's Buffer and pipetting up and down twice to complete the 1:1000 dilution. 45 μL of the diluted blood was then added to an amber tube (Sarstedt Catalog No 72.694.034 or equivalent) containing 5 μL of CD61-Cy5 or 10E5-Cy5 (5μg/mL) and incubated at room temperature for 20 minutes.

40μL of the diluted blood stained with a pan-platelet marker was placed in the well of a plastic capillary (Catalog No VC120, Biometric Imaging, Mountain View, CA) and the fluorescence intensity and number of events within the platelet size gate was determined on the IMAGN2000 instrument (Biometric Imaging, Mountain View, CA).

Example 6: Use of a volumetric capillary cytometry system to measurement of soluble P-selectin:

The IMAGN2000™ volumetric capillary cytometry system was used to measure soluble P-selectin. The preferred embodiment is using a bead-based format. The sample for this example is prepared in the same way as the sample that was prepared in Example 2, discussing the ELISA method of determining soluble P-selectin. Rather than coating a microtiter plate as in Example 2, the technician coats the polystyrene beads with the anti-P-selectin antibody, W40. The beads, also called polystyrene sulfated microparticles, (9,7μm) were passively coated at 0.5 x the available particle surface area with the P-selectin specific antibody, W40. The beads were diluted in 30 mM phosphate buffered saline, 1% BSA, 0.01% Tween 20 such that the solids comprised 0.01% of the assay volume. Soluble P-selectin produced by a transfected human kidney cell line (293 cells) was added to the beads in diluent at a range of concentrations. S12-Cy5, a fluorescently labeled anti-P-selectin antibody, was added at a final concentration of 2.5 μg/mL in the assay and incubated with shaking for two

hours at room temperature. Rather than putting the microtiter plate in the plate reader as in Example 2, the technician places the assay mixture containing the beads in the capillary of the IMAGN2000™ machine. 40 μL of the assay mixture was placed in the well of a plastic capillary (Catalog No VC120, Biometric Imaging , Mountain View, CA) and the fluorescence intensity and number of events within the platelet size gate was determined on the IMAGN2000 instrument (Biometric Imaging, Mountain View, CA).

Example 7: Measurements and sensitivity of P-selectin in the presence of a platelet agonist using a volumetric capillary cytometry system:

The article of manufacture, IMAGN2000™, provides the capability to detect in a sample of whole blood, the number of platelets that are activated and therefore are expressing P-selectin on their surface (membrane). Activated platelets are detected by the addition of a labeled P-selectin specific Mab (in this embodiment a cocktail of S12-Cy5 and W40-Cy5). A fixative is finally added to the whole blood to insure that *in vitro* platelet activation does not occur. Blood which has been incubated with labeled P-selectin Mab(s) and fixed is then placed into a capillary and a predetermined volume of the blood is scanned by the optical (laser) mechanism of the instrument. A size range that allows for the discrimination of different cell types based on size can be pre-programmed into the instrument. Within the pre-determined size range,

every fluorescent event of magnitude sufficiently above background fluorescence is recorded as one event. When whole blood is subjected to increasing concentrations of platelet activation agonist, an increasing number of platelets is activated to the degree that their fluorescence intensity (signal) is sufficiently above background (noise) such that the ratio of signal to noise qualifies them to be counted by the instrument as an event. In a series of experiments performed with the instrument, the platelet activation agonist PMA (phorbol myristate acetate) was added in increasing quantities to whole blood. The results validate the utility of the instrument in assessing the number of activated platelets present in a whole blood sample. In the absence of agonist, four positive event (platelets) were determined to be in the approximately 5 μL of blood diluted 1:25 that was scanned by the laser. At 5nM PMA, eight activated platelets were detected. At 40 nM PMA 120 activated platelets were detected and at 500nM PMA 965 activated platelets were counted. The current model of this instrument counts a maximum of 1000 events. In addition to the detection of the number of activated platelets, using a labeled P-selectin specific Mab(s), a second Mab which is specific for a surface molecule common to all platelets (a pan platelet marker) is also added to the whole blood. In this particular embodiment, the marker used is CD61-Cy5. Each platelet will have sufficient CD61-Cy5 on its membrane to be detected as an event (high enough signal to noise

ratio). The number of events positive for CD61-Cy5 when adjusted for the volume scanned and the dilution factor of the blood will provide a count of the number of platelets in the whole blood. Dividing the number of events (platelets) positive for P-selectin by the total number of platelets will result in the percent positive platelets. The percent for normal states and for activated platelet states has been established by flow cytometry. The percent positive platelets derived from the current instrument will initially be correlated with a similar calculation derived from flow cytometric determinations on the same sample to establish the substantial equivalence of the two methods.

Example 8: Measurement of P-selectin using a bead format in a volumetric capillary cytometry system:

The article of manufacture, IMAGN2000, provides the capability to detect and quantify the amount of soluble P-selectin present in a sample of platelet poor plasma (PPP). In this embodiment of the assay, 9.7 μM latex beads were coated (covalently or passively) with a P-selectin specific Mab. The coating of the beads was carefully controlled so that a uniform amount of P-selectin Mab was present on each bead. A specified number of beads were incubated in PPP containing soluble P-selectin. Simultaneously with the addition of the beads, a Cy5 labeled P-selectin specific Mab binding to a different site on soluble P-selectin from the site bound by the Mab used to coat the bead, or a Cy5 labeled polyclonal anti-P-selectin antibody preparation is

added to the PPP. At the end of the incubation period, the plasma containing the beads is placed in a capillary and a predetermined volume of plasma is scanned by the optical (laser) mechanism of the instrument. A size determination that includes the 9.7 μM beads is pre-set within the program of the instrument. Within that size window, the fluorescent intensity of all events which are sufficiently fluorescent (above background) will be calculated. The fluorescent intensity of the beads will be directly proportional to the amount of soluble P-selectin bound by the surface Mab and detected by the labeled P-selectin antibody(ies). In order to quantify the amount of soluble P-selectin which correlates with a specific fluorescence intensity, a series of beads will be coated with increasing and carefully determined amounts of isolated recombinant soluble P-selectin and then incubated with the Cy5 labeled anti-P-selectin antibody used in the assay. In this way, a standard curve of fluorescence intensity corresponding to soluble P-selectin concentration is established. The amount of soluble P-selectin bound to beads incubated with the test plasma can then be determined from the standard curve solely by determining the fluorescence intensity of the beads. Initial assays with this method show that when known quantities of isolated recombinant soluble P-selectin are spiked into human plasma and incubated with anti-P-selectin Mab (W40) coated beads in the presence of Cy5 labeled S12, the fluorescent intensity of the beads increases linearly with the increased concentration of soluble P-selectin.

This method is an alternative way to measure soluble P-selectin that will provide results comparable to the reference method which is the ELISA described in this document.

Example 9: Measurement of Soluble P-selectin For Large Scale Study:

A P-selectin EIA kit was produced by Centocor Diagnostics, Inc., Malvern, Pennsylvania, (the "Centocor Diagnostics P-selectin EIA"). This P-selectin EIA was an *in vitro* test for the quantitative determination of soluble P-selectin in plasma.

PRINCIPLE OF THE PROCEDURE

The Centocor Diagnostics P-selectin EIA is based on the classical single step ELISA sandwich assay using streptavidin technology. Soluble P-selectin from the patient's specimen reacts with the biotinylated anti-P-selectin antibody, which in turn is bound by the streptavidin coated walls of the 96-well plate. Simultaneously HRP-conjugated anti-P-selectin antibody binds to the immobilized complex. The quantity of antibody complex formed is a measure of the soluble P-selectin content of the specimen. The unbound conjugate is removed along with other plasma components during the wash step. Surface-bound enzyme-labeled P-selectin is identified by reaction with the substrate, tetramethylbenzidine (TMB). The intensity of the colored

reaction product is directly proportional to the amount of soluble P-selectin present.

REAGENTS

1.	Coated Microwell Strips Plastic microtitration wells coated with Streptavidin	1 Plate (96 wells)
2.	High Control (see container label for assigned value) Human plasma containing soluble P-selectin and 0.1% ProClin 300 as preservative.	1 Vial, 0.5 ml
3.	Low Control (see container label for assigned value) Human plasma containing soluble P-selectin and 0.1% ProClin 300 as preservative.	1 Vial, 0.5 ml
4.	Standards 1-5 (see container label for assigned value) soluble P-selectin supernatant and 0.1% ProClin 300 as preservative.	1 Vial, 0.5 ml
5.	Biotinylated Antibody Monoclonal antibody diluted in Assay Buffer	1 Vial, 1.0 ml

6. HRP-Conjugate 1 Vial, 1.0 ml
Monoclonal antibody diluted
in Phosphate buffered saline, 1.0% BSA,
0.05% Tween, and 0.1% Proclin 300
as a preservative

7. Assay Buffer 1 Bottle, 50.0 ml
Phosphate buffered saline, 1.0% BSA,
0.05% Tween, 25 g/ml B2TT,
0.00025% Sodium Azide and 0.1%
Proclin 300 as a preservative

8. Wash Buffer (10X), Phosphate buffered saline, 0.5% Tween 1 Bottle,100ml

9. Instructions for use 1 Set of Instructions

STORAGE AND STABILITY

The Centocor Diagnostics P-selectin EIA Kit should be stored at 2-8°C.

Do not use the components beyond the expiration date on the label.

Indications of Deterioration.

The Centocor Diagnostics P-selectin EIA may be considered to have deteriorated if:

1. The kit fails to meet the required QC criteria for a valid test.
2. Liquid reagents become visibly cloudy or develop precipitates.

3. If the desiccant located in the plate pouch changes color from purple to pink, the plate should not be used.

SPECIMEN COLLECTION AND STORAGE

Centocor Diagnostics P-selectin EIA is intended for use with platelet-poor plasma prepared from whole blood collected in Becton Dickinson Diatube-H Vacutainers containing the anticoagulant C.T.A.D. The blood must be drawn into the Vacutainer using a 19-gauge or larger needle. The Vacutainer is inverted gently two times to mix. Further agitation of the specimen must be avoided. Clotted or excessively hemolyzed samples are unacceptable. Store the whole blood at room temperature (18-25°C). Preparation of platelet poor plasma by centrifugation should be performed within a preparation window of 60 minutes after the specimen has been drawn. However, plasma prepared after the 60 minute preferred processing window may still be used in performing the assay.

Centrifuge the whole blood at room temperature for 15 minutes at 1800 X g. Transfer the clear, straw-colored platelet-poor plasma from above the cell pellet to polypropylene freezer vials using a plastic transfer pipette. Store the plasma at 2-8° C for no longer than 72 hours prior to performing the assay. If extended storage is required, freeze the plasma at $\leq$-70° C.

Note: Assure that the centrifugation process you are using produces plasma with a platelet concentration below the level of detection of an automated blood cell counter (such as a Coulter counter).

MATERIALS SUPPLIED

Centocor Diagnostics P-selectin EIA, 96 Tests (Refer to REAGENTS for a list of materials provided.)

Sufficient quantities of the following accessories are provided to perform 96 tests. The following items will be shipped under separate cover.

1. Reagent reservoirs
2. Adhesive cover sealers
3. TMB Substrate Solution, Sigma, Cat. No. T8665
4. Sulfuric Acid, 1N Volumetric Solution (Stop Solution), Baker, Cat. No. 5642-02
5. 1.5 ml Eppendorf microcentrifuge tubes, or 12x75 snap cap polypropylene tubes
6. Diatube-H Vacutainers containing the anticoagulant C.T.A.D., Becton Dickinson, Cat. No. 367015

MATERIALS REQUIRED (BUT NOT SUPPLIED)

1. Disposable tip micropipettes to deliver 25 µl, 50 µl, 100 µl, 1000 l (single and multichannel pipettes required).
2. Deionized water.

3. Labware consisting of, at least, 1 L graduated cylinder, 15 ml polypropylene tubes, 10 ml and 25 ml disposable plastic pipettes.
4. Absorbent paper towels.
5. Automatic microtitration plate washer or laboratory wash bottle.
6. Microtitration plate reader with 450 and 650 nm filter.
7. Latex gloves, safety glasses and other appropriate protective garments.
8. Biohazard infectious waste containers.
9. Safety pipetting devices for 1ml or larger pipettes.
10. Timer.
11. 19 gauge or larger venipuncture needles

REAGENT PREPARATION

Bring kit reagents to room temperature (18-25° C) before preparation or use of working solutions. Bring coated plate to room temperature and then remove from foil pouch just prior to use.

1. Wash Solution

Add 1 bottle (100 ml) of concentrated Wash Buffer (10X) to 900 mL of deionized water. Mix until homogeneous.

2. Biotinylated Antibody/ HRP conjugate solution.

Transfer 9 ml of Assay Buffer into a 15 ml tube. To the Assay Buffer add 0.5 ml of Biotinylated Antibody and 0.5 ml of HRP-Conjugate. Prepare within 10 minutes of addition to plate.

3. **Controls: High and Low**

Add 475μl of Assay Buffer to each of two labeled 1.5 ml Eppendorf tubes or 12x75 mm snap cap polypropylene tubes. Add 25 μl of High Control to one tube and 25 μl of Low Control to the other tube, cap and mix.

TEST PROCEDURE

Procedural Warnings

The Centocor Diagnostics P-selectin EIA kit contained the following procedural warnings:

1. This kit should be used in strict accordance with the instructions in the Package Insert.
2. Centocor Diagnostics P-selectin EIA kits contain reagent systems which are optimized and balanced for each kit lot. Do not interchange reagents from kits with different lot numbers. Do not interchange vial caps or stoppers either within or between kits.
3. Do not use Centocor Diagnostics P-selectin EIA kits after the expiration date printed on the outer carton label.
4. Do not cross-contaminate reagents. Always use fresh pipette tips when drawing from stock reagent bottles.
5. Always use clean, preferably disposable, labware for all reagent preparation.
6. Allow the foil pouch containing the plate to equilibrate at room temperature before opening. This avoids condensation on the inner surface of the bag

which may contribute to a deterioration of coated plates.

7. Reagents should be dispensed with the tip of the micropipette touching the side of the well at a point near mid-section.

8. Always keep the upper surface of the plates free from excess fluid droplets. Reagents and buffer overspill should be blotted dry on completion of the manipulation.

9. Do not allow the wells to completely dry during the assay.

10. Ensure that the TMB Substrate Solution, which may freeze at 2-8°C, is completely thawed before use. Repeated freezing and thawing does not affect the stability of TMB. TMB must be at room temperature before use.

11. Disposal or decontamination of fluid in the waste reservoir from either the plate washer or trap for vacuum line in the manual system should be in accordance with guidelines set forth in the Department of Labor, Occupational Safety and Health Administration, occupational exposure to blood-borne pathogens; final rule (29 CFR 1910, 1030) FEDERAL REGISTER, pp 64176-84177, 12/6/91.

12 Automatic or semi-automatic EIA processors or liquid handling systems should be qualified specifically for use with Centocor Diagnostics P-selectin EIA by

demonstration of equivalence to the manual processing methods.

13. Consistent with good laboratory practice, it is recommended that all pipetting devices (manual or automatic) are regularly calibrated according to the manufacturer's instructions.

14. Care must be taken to ensure that specimens are dispensed correctly to each test well. If a specimen is inadvertently not added to a well, the result for that well will be non-reactive, regardless of the actual result of the specimen.

Wash Cycle

Automated Method

Efficient rinsing to remove uncomplexed sample components is a fundamental requirement of enzyme immunoassay procedures. Centocor Diagnostics P-selectin EIA utilizes one standard three-wash cycle. Automatic plate washers may be used provided they meet the following criteria:

1. All wells are completely aspirated.
2. All wells are filled to the rims during the wash cycle.

The plate washer must be well maintained to prevent contamination from previous use. Manufacturer's cleaning procedures must be followed diligently.

For each wash cycle, the machine should be set to three consecutive washes. On completion of the cycle, invert the plate and tap firmly on absorbent paper towels. Check for

any residual Wash Buffer in the wells and blot dry the upper surface of the wells with a paper towel.

Manual Method

Aspirate each well using a multichannel aspirating pipette attached to a vacuum source. Rinse plate by adding 300 L of Wash Buffer to each well. Take care not to overfill the well. Remove Wash Buffer by aspiration and repeat the wash procedure two additional times.

Preparation for the Assay

Specimen Preparation

See Specimen Collection and Storage Section.
Specimens must be pre-diluted as follows:

Add 475μl of Assay Buffer to a 1.5 ml Eppendorf tubes or 12x75 snap cap polypropylene tubes. Add 25 μl of specimen to the Eppendorf tube or 12x75 snap cap polypropylene tube, cap and mix.

Kit Controls Preparation

Kit controls must be prediluted as follows:

Add 475μl of Assay Buffer to each of two 1.5 ml Eppendorf tubes or 12x75 snap cap polypropylene tubes. Add 25 μl of High Control to one tube and 25 μl of Low Control to the other tube, cap and mix.

The kit controls should be included on each microtitration plate. The Low and High Controls should always be tested in triplicate.

Kit Standards

The kit standards are to be used straight from the bottle. No further dilution is required

The kit standards and Assay Buffer should be included on each microtitration plate. The kit standards and Assay Buffer should always be tested in triplicate.

Wash Buffer

Prepare working-strength Wash Buffer by diluting 100 ml Wash Buffer (10X) concentrate with 900 ml of deionized water.

Assay Procedure

1. Allow kit reagents to reach room temperature (18-25° C).
2. Prepare wash buffer, controls, and specimens as described above.
3. Label plate appropriately.
4. Pipette 50 µl of the 5 standards, assay buffer (as a zero standard), low and high controls (pre-diluted) on the plate run in triplicate. Pipette 50 µl of the pre-diluted specimens in duplicate.
5. Prepare biotinylated antibody/HRP-conjugate solution as described above, within 10 minutes of addition to plate.

6. Pipette 50 l Biotinylated Antibody/HRP Conjugate solution to each well containing standards, controls, assay buffer, and specimens.

7. Cover the plate with a cover seal and tap the 96-well plate gently to ensure thorough mixing of the reagents.

8. Incubate in the dark at room temperature (18-25°C) for 3 hours +/- 10 minutes. Record the assay start time, stop time, and room temperature.

9. Transfer the previously prepared working solution of Wash Buffer to the reservoir of an automatic 96-well plate washer or other suitable container.

10. At the end of the 3 hour incubation period, wash the plate 3X with the Wash Buffer. Remove any excess Wash Buffer by tapping plate on a paper towel.

11. Using a multichannel pipette, add 200 l TMB Substrate Solution to each well of the plate. Incubate in the dark at room temperature (18-25°C) for 30 ± 2 minutes. Record the assay start time, stop time, and room temperature.

12. Halt the color development by adding 100 µl Stop Solution (1N Sulfuric acid) to each well of the plate. Tap plate gently to ensure adequate mixing of the Stop Solution. Store plate in the dark until read.

13. Read plate at 450 and 650 nm within 30 minutes of addition of the Stop Solution. Subtract the 650 nm reading from the 450 nm reading.

QC Criteria

1. CV percent on the OD of standards (1▲5), controls, and samples must be < 15%.
A single well in the standard curve may be dropped to correct a high CV.
A single well in one control sample may be dropped to correct a high CV.
When dropping the single well value, the point farthest from the mean of the three well values is the point to be dropped.

2. The O.D. of the standards, beginning with standard 1, must decrease as the standard number increases.

3. The mean O.D. of Standard 1 must be between 2.0 and 4.0 absorbance units.

4. The mean O.D. of the Assay Buffer, Standard 6, must be less than 0.060 OD units.

5. Both the High and Low Controls must read within the label range.

6. Repeat any failed assay in total. Repeat any specimen that did not meet QC criteria (high CV or value greater than Standard 1) in another assay.

Evaluation

1. Generate a standard curve using standards 1-5 and Assay Buffer (as a 0 ng/ml standard) and fit data using a quadratic regression method.

2. Report values for each valid assay.

3. It is not possible to extrapolate the standard curve above 850 ng/mL. Therefore, specimens with values greater than 850 ng/mL must be diluted further and reassayed. Specimens above 850 ng/mL must be diluted 1:100 in Assay Buffer and retested as described in Procedure for Assay of Patient Samples with Greater than 850 ng/mL (Below).

PROCEDURE FOR ASSAY OF PATIENT SPECIMENS WITH GREATER THAN 850 NG/ML

Accurate determination of soluble P-selectin assay values for specimens which exceed 850 ng/mL requires that the specimen be further diluted and reassayed. A 1:100 dilution from the original specimen should be prepared in Assay Buffer. Mix thoroughly before assaying. Perform the assay according to Assay Procedure. Obtain results by multiplying the value obtained in ng/mL from the standard curve by the additional dilution factor above 1:20.

Example 10: Measurement of Platelet P-selectin for Large Scale Study.

A P-selectin FLOW test kit was produced by Centocor Diagnostics, Inc., Malvern, Pennsylvania, (the "Centocor Diagnostics P-selectin EIA"). This P-selectin FLOW Test Kit was an *in vitro* test for the quantitative determination of platelet-bound P-selectin in whole blood. This kit is for investigational use only.

PRINCIPLE OF PROCEDURE

The Centocor Diagnostic P-selectin FLOW Test Kit utilizes standard two-color flow cytometry technology to detect platelet-bound P-selectin on the surface of activated platelets in a whole blood specimen. The test involves the use of phycoerythrin (PE) conjugated monoclonal antibody specific for the CD41 antigen, present on all platelets. The use of this reagent defines the platelet population. The test also includes the use of a fluorescein isothiocyanate (FITC) conjugated monoclonal antibody specific for CD62 (P-selectin) which is detected only on the surface of activated platelets and not on the surface of unactivated platelets. The use of the two antibodies allows for the determination of the percent of total platelets which are in the activated state and the mean channel fluorescence of the FITC-labeled P-selectin MAb. The test consists of the evaluation of an unstained whole blood specimen to determine any unusual contribution of autofluorescence by the particular specimen. The

negative control in the assay is a cocktail consisting of an antibody of the same isotype as the CD62 specific MAb but one which does not have binding specificity for platelets. The test reagent in the assay is a pan-platelet -PE, P-selectin-FITC MAb cocktail. The pan-platelet MAb in both the negative control and the test reagent acts as a marker binding to platelets and showing that the cocktails were added to samples even if the platelets in the sample are unactivated and no P-selectin expression is observed.

REAGENTS

Centocor Diagnostics P-selectin FLOW Test Kit reagents supplied in this kit are for investigational use only.

1. Reagent A 1 vial, 5.0 mL
   Buffer, contains 0.1% Sodium Azide as a preservative.
   For the preparation of unstained platelet samples.
2. Reagent B 1 vial, 5.0 mL
   FITC-conjugated isotype control monoclonal antibody with PE-conjugated pan-platelet monoclonal antibody. Contains 0.1% Sodium Azide as a preservative.
   For the preparation of a two-color green fluorescence negative control and red fluorescence platelet identifier.
3. Reagent C 1 vial, 5.0 mL
   FITC-conjugated anti-P-selectin monoclonal antibody with PE-conjugated pan-platelet monoclonal antibody. Contains 0.1% Sodium Azide as a preservative.

For the preparation of a two-color green fluorescence P-selectin marker and red fluorescence platelet identifier.

4. Diluent 1 Bottle, 100 mL

Buffer, Contains 0.1% Sodium Azide as a preservative.

5. Instructions for use 1 Set of Instructions

STORAGE AND STABILITY

All reagents should be stored at 2-8°C and should not be used beyond the expiration date on the label.

Indications of Deterioration.

The Centocor Diagnostics P-selectin FLOW Test Kit may be considered to have deteriorated if:

1. The kit fails to meet the required QC criteria for a valid test.

2.- Liquid reagents become visibly cloudy or develop precipitates.

INSTRUMENT SET-UP

1. Initial instrument set-up shall be performed by an individual authorized by Centocor Diagnostics according to an approved protocol. Changes to the instrument settings are only needed after major instrument failure and/or subsequent repair.

2. Quality Control of the instrument should be performed daily using appropriate commercially

available fluorescent beads listed in this insert. Any deviations from expected QC results should be addressed prior to proceeding with sample run.

SAMPLE RUN ON INSTRUMENT

1. With the instrument set up per protocol, dilute sample prepared with Reagent A (unstained) in PBS, so as to obtain a flow rate of 800 - 1000 events per second (e/s). Event rates higher than 1000 e/s can increase platelet coincidence with RBCs (red blood cells).

2. Acquire and save data from all events to guarantee collection of a minimum of 10,000 platelets based upon a platelet gate set on the FSC vs. SSC dot plot. During the acquisition, should the RBCs begin to shrink and interfere with the accurate counting of platelets, prepare a freshly diluted sample and continue the collection.

3. Dilute sample prepared with Reagent B (isotype/pan-platelet cocktail) in PBS, so as to obtain a flow rate of 800 - 1000 e/s.

4. Acquire and save data from all events to guarantee collection of a minimum of 10,000 platelets based upon a platelet gate set on the PE-positive platelets of a SSC vs. FL2 dot plot. Exclude the small percentage of PE-positive events having the same SSC as the RBCs. Increasing the flow rate above 1000 e/s increases the

number of events in this latter population, therefore, regulate the flow rate for each sample.

5. Repeat previous two steps for sample stained with Reagent C (anti-P-selectin/pan-platelet cocktail).

6. Analyze the unstained sample by setting a FSC vs. SSC platelet gate. Then display a FL1 vs. FL2 dot plot (log scale) of the same sample gated on this platelet gate. Set the quad stat cursors at the Centocor approved settings historically observed with unstained platelets from apparently healthy donor samples processed using the Centocor Diagnostics P-selectin Compensation Kit. Display % gated events in each quadrant using quadrant statistics.

7. Analyze Reagent B stained sample by setting a SSC vs. FL2 platelet gate. Display a single parameter histogram of FL1(log scale) gated on the platelet gate. Set a histogram statistical marker such that 1 ± 0.1% of the events are to the right of that marker when histogram statistics are displayed. Include the geometric mean of the entire histogram in the statistics window as well.

8. Analyze Reagent C stained sample by setting a SSC vs. FL2 platelet gate. Display a single parameter histogram of FL1 (log scale) gated on the platelet gate. Copy the same histogram statistical marker set for Reagent B to this histogram and determine % positivity using histogram statistics. (If available with the analysis software, the two histograms from

Reagent B and Reagent C stained samples can be overlaid for optimal viewing and comparison.)

9. If the computer software has the ability to perform "acquisition to analysis" plots providing instant results after completion of each sample collection, these may be used instead of "acquisition" plots requiring analysis after completion of the entire sample run.

SPECIMEN COLLECTION AND STORAGE

Centocor Diagnostics P-selectin FLOW Test Kit is intended for use with whole blood collected in Becton Dickinson Diatube-H Vacutainer containing the anticoagulant C.T.A.D.. The blood must be drawn into the Vacutainer using a 19-gauge or larger needle. The Vacutainer is inverted gently two times to mix. Further agitation of the specimen must be avoided. Clotted or excessively hemolyzed samples are unacceptable. Store the whole blood at room temperature (18-25°C). Assays should be performed within an assay window of 30 minutes after the specimen has been drawn . However, specimens processed after the 30 minute preferred assay window may still be used in performing the assay.

Blood samples collected for the FLOW assay can be further processed into platelet-poor plasma for assaying in the Centocor Diagnostics P-selectin EIA. Preparation of platelet-poor plasma, as detailed in the EIA product insert, should begin within 60 minutes of blood collection.

MATERIALS SUPPLIED

Centocor Diagnostics P-selectin FLOW Test Kit, 100 Tests (Refer to REAGENTS for a list of material provided.)

Sufficient quantity of the following accessories are provided (under separate cover) to perform 100 tests:

1. 10% Paraformaldehyde, EM Grade Formaldehyde Methanol-free, Electron Microscopy Sciences, Cat. No. 15712-S
2. Eppendorf tube, 1.5 mL, VWR Cat. No. 21008-959, or equivalent
3. Amber tube, 1.5 mL, with cap, Sarstedt Cat. No. 72.694.034, or equivalent
4. Diatube-H (C.T.A.D.)Vacutainer , Becton Dickinson Cat. No. 367015
5. Quantum 24 Beads, Flow Cytometry Standards Corporation, Cat. No. 824
6. QC3 Microbeads Beads, Flow Cytometry Standards Corporation, Cat. No. 847C
7. Full Spectrum Microbeads, Flow Cytometry Standards Corporation, Cat. No. 855C
8. Dulbecco's Phosphate Buffered Saline 1X, without Calcium and Magnesium, pH = 7.3-7.4, BioWhittaker, Cat. No. 12-512F, or equivalent

MATERIALS REQUIRED (BUT NOT SUPPLIED)

1. Micropipettors (Gilson P20, P200, and P1000, or equivalent) with tips

2. Latex gloves, safety glasses and other appropriate protective garments
3. Timer
4. Flow Cytometer
5. 19 gauge or larger venipuncture needle for use with Vacutainer
6. Sheath fluid, FACSFlow Saline Solution, Becton Dickinson Cat. No. 340398, or equivalent
7. pH meter and pH standards
8. HCl solution for pH
9. Biohazard infectious waste containers
10. Aluminum foil
11. Flow Cytometer Bead Controls, CaliBrite Beads, Becton Dickinson Cat. No. 349502, or equivalent daily quality control beads

REAGENT PREPARATION

Bring all reagents to room temperature (18-25° C) before preparation or use of working solutions.

2% Paraformaldehyde

Add 4.0 mL Diluent to a labeled 15 mL centrifuge tube. Add 1.0 mL 10% Paraformaldehyde to the tube. Cap the tube, mix by vortexing, cover tube with foil, and store at 2-8°C, in the dark. Expiration date of 2% Paraformaldehyde is 1 week after preparation. Follow precautions when handling and disposing of Paraformaldehyde.

Dulbecco's Phosphate Buffered Saline (1X) pH = 7.3 - 7.4 [PBS]

Determine pH of PBS using a calibrated pH meter. If pH result is >7.4, lower the pH using HCl to the range of 7.3 - 7.4. Should the pH be or drop below 7.3 during this process, discard the supplied PBS and contact Centocor Diagnostics. **Do not attempt to raise the pH to 7.3-7.4 using NaOH.**

TEST PROCEDURE

Procedural Warnings

The Centocor Diagnostics P-selectin FLOW Test kit contained the following procedural warnings:

1. This kit should be used in strict accordance with the instructions in the Package Insert.
2. The Centocor Diagnostics P-selectin FLOW Test Kit must be used in conjunction with the Centocor Diagnostics P-Selectin Compensation Kit indicated on the kit box label. In order to obtain proper results, the kits must be used with the prescribed matched complementary kit.
3. Centocor Diagnostics P-selectin FLOW Test Kits contain reagent systems which are optimized and balanced for each kit lot. Do not interchange reagents from kits with different lot numbers. Do not interchange vial caps or stoppers either within or between kits.

4. Do not use Centocor Diagnostics P-selectin FLOW Test Kits after the expiration date printed on the outer carton label.

5. Do not cross-contaminate reagents. Always use fresh pipette tips when drawing from stock reagent bottles.

6. Always use clean, polypropylene, disposable, labware for all reagent preparation.

7. Reagents should be dispensed with the tip of the micropipette touching the bottom of the tube.

8. Disposal or decontamination of fluid in the waste reservoir from the flow cytometer should be in accordance with guidelines set forth in the Department of Labor, Occupational Safety and Health Administration, occupational exposure to blood-borne pathogens; final rule (29 CFR 1910, 1030) FEDERAL REGISTER, pp 64176-84177, 12/6/91.

9. Consistent with good laboratory practice, it is recommended that all pipetting devices (manual or automatic) are regularly calibrated according to the manufacturer's instructions.

10. Care must be taken to ensure that specimens are dispensed correctly. If a specimen is inadvertently not properly added, the result for that specimen will be negative, regardless of the actual result of the specimen.

11. Agitation of unfixed blood beyond the mixing specified in this procedure may cause artifactual *in vitro* activation. Never vortex an unfixed sample of

blood. Avoid processing samples under a cold draft such as an air conditioning vent.

Staining Procedure

1. Remove Diluent, Reagents A, B and C and the previously diluted 2% Paraformaldehyde from refrigerator. Allow to equilibrate to room temperature (18-25°C). Label appropriate tubes.
2. Obtain a patient's specimen (see Specimen Collection and Storage section).
3. Label 3 amber screw-cap tubes with reagent name, patient number and any other appropriate identification.
4. Add 40 L of reagents A, B, and C to the bottom of the appropriately labeled tubes.
5. Label one Eppendorf tube for the dilution of the patient's whole blood specimen.
6. Add 450 L of Diluent, to the Eppendorf tube.
7. Gently invert the whole blood tube to mix. Transfer 50 l whole blood to the Eppendorf tube.
8. Mix the diluted whole blood specimen by inverting the Eppendorf tube gently, twice.
9. Transfer 60 L of the diluted whole blood specimen to the bottom of each of the 3 amber tubes containing staining reagents A - C. (Change pipette tip after each transfer.) Do not mix.

10. Cover the amber tubes with foil and incubate the tubes at room temperature (18 - 25°C) for 15 ± 1 minutes.

11. Upon completion of the reagent incubation, add 100 L 2% Paraformaldehyde to the bottom of each of the amber tubes. (Change pipette tip after each transfer.) Do not mix.

12. Cap each tube and incubate them at room temperature (18 - 25°C) for 30 minutes (no longer than 2 hours).

13. After this fixation incubation, store the capped reaction tubes at 2-8°C for up to 72 hours prior to flow cytometric analysis.

QC CRITERIA

1. For all Reagent A samples, the quadrant statistics of the upper right quadrant of a FL1/FL2 dot plot (log scale) gated on the platelet population of the FSC/SSC dot plot (log scale) should show ≤ 2% of all gated events with ≥98% of the gated platelet population falling in the lower left quadrant (first decade). Report the percent platelets in the lower left quadrant, on the data transmittal form.

2. Both Reagent B and C samples must show positivity of FL2 10^2 for the pan-platelet conjugate. Absence of this result indicates either reagent degradation or absence of the reagent in the prepared sample and thus disqualifies the sample from further analysis.

RESULTS

Results are reported as per sponsor's protocol as % platelet-bound P-selectin positive based upon a single parameter histogram (FL1) gated on platelets which are positive for the pan-platelet marker (SSC vs. FL2). Results passing the QC criteria suggest that the assay was performed according to instructions and that the reagents have performed as expected.

EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

CLAIMS

What is claimed is:

1. A method for diagnosing the presence or absence of a thrombotic disorder in a patient comprising assessing the level of membrane bound P-selectin in a sample comprising platelets from said patient, wherein an elevated P-selectin level is indicative of a positive diagnosis.

2. The method of Claim 1, wherein the thrombotic disorder is selected from a group consisting of myocardial infarction, unstable angina, stroke, pulmonary embolism, transient ischemic attack, deep vein thrombosis, and thrombotic re-occlusion subsequent to a coronary intervention procedure.

3. A method for diagnosing the presence or absence of a thrombotic disorder in a patient comprising assessing the level of soluble P-selectin in a sample comprising aqueous blood components from said patient, wherein an elevated P-selectin level is indicative of a positive diagnosis.

4. The method of Claim 3, wherein the thrombotic disorder is selected from a group consisting of myocardial infarction, unstable angina, stroke, pulmonary

embolism, transient ischemic attack, deep vein thrombosis, and thrombotic re-occlusion subsequent to a coronary intervention procedure.

5. A method for diagnosing the presence or absence of a thrombotic disorder in a patient comprising:

a) assessing the level of membrane bound P-selectin in a sample comprising platelets from said patient; and

b) assessing the level of soluble P-selectin in a sample comprising aqueous blood components from said patient,

wherein an elevated P-selectin level as assessed in step (a) and/or in step (b) is indicative of a positive diagnosis.

6. The method of Claim 5, wherein the thrombotic disorder is selected from a group consisting of myocardial infarction, unstable angina, stroke, pulmonary embolism, transient ischemic attack, deep vein thrombosis, and thrombotic re-occlusion subsequent to a coronary intervention procedure.

7. The method of Claim 6, wherein step (a) and step (b) are conducted essentially simultaneously.

8. A method for diagnosing the presence or absence of myocardial infarction or angina in a patient comprising:

a) assessing the level of membrane bound P-selectin in a sample comprising platelets from said patient; and

b) assessing the level of soluble P-selectin in a sample from said patient,

wherein an elevated P-selectin level as assessed in step (a) and/or in step (b) is indicative of a positive diagnosis.

9. The method of Claim 8, wherein step (a) and step (b) are conducted essentially simultaneously.

10. A method for determining the presence or absence of a thrombotic disorder in a patient comprising:

a) obtaining a blood sample from said patient,

b) assessing the level of membrane bound P-selectin in a sample comprising platelets from said patient, and

c) assessing the level of soluble P-selectin in a sample from said patient

wherein assessing said levels of P-selectin further comprises contacting said sample with at least one antibody specific to P-selectin, and detecting said levels of P-selectin; wherein an elevated P-selectin

level as assessed in step (b) and/or in step (c) is indicative of a positive diagnosis.

11. A method of claim 10, wherein said antibody is detectably labeled.

12. A method of claim 10, wherein assessing the level of membrane bound P-selectin further comprises contacting said sample with at least one platelet specific antibody which is specific to a receptor on essentially all platelets, said platelet specific antibody is detectably labeled.

13. A method of claim 10, wherein assessing the level of soluble P-selectin further comprises contacting said sample with a second antibody specific to P-selectin or a complex between an antibody and P-selectin.

14. A method of differentiating between a thrombotic disorder and a symptomatic, but non-thrombotic disorder in a patient comprising:

a) assessing the level of membrane bound P-selectin in a sample comprising platelets from said patient; and

b) assessing the level of soluble P-selectin in a sample from said patient,

wherein an elevated P-selectin level as assessed in step (a) and/or in step (b) is indicative of a thrombotic disorder.

15. A method of determining endogenous platelet activation comprising:

a) assessing the level of membrane bound P-selectin in sample comprising platelets from said patient;

b) assessing the level of soluble P-selectin in sample comprising blood components from said patient,

wherein an elevated P-selectin level as assessed in step (a) and/or step (b) is indicative of endogenous platelet activation.

16. A method for diagnosing the presence or absence of a thrombotic disorder in a patient comprising:

a) assessing a level of at least one platelet activation marker, and

b) assessing a level of at least one thrombin generation marker,

wherein the level of the platelet activation marker and the level of the thrombin generation marker is indicative of the presence or absence of a thrombotic disorder.

17. The method of claim 16, further comprising assessing a level of at least two platelet activation markers.

18. The method of claim 17, wherein assessing the level of at least two platelet activation markers comprises:

a) assessing the level of membrane bound P-selectin in a sample comprising platelets from said patient;

b) assessing the level of soluble P-selectin in a sample comprising blood components from said patient.

19. The method of claim 18, wherein the thrombin generation marker is selected from a group consisting of creatine kinase muscle brain, D-Dimer, F1.2, thrombin anti-thrombin, soluble fibrin monomer, fibrin peptide A, myoglobin, thrombin precursor protein, platelet monocyte aggregate and troponin.

20. The method of claim 19, further comprising assessing a result of a diagnostic test for diagnosing thrombotic disorders.

21. The method of claim 20, wherein the diagnostic test is an electrocardiogram.

22. The method of claim 21, wherein the assessment of said levels occurs in a range between about zero and 8 hours from the onset of the thrombotic disorder.

23. The method of claim 22, wherein the positive diagnosis occurs in a range between about 1 and 6 hours.

24. A method for diagnosing the presence or absence of a cardiac, thrombotic disorder in a patient comprising:

a) assessing a level of at least one platelet activation marker, and

b) assessing a result of at least one diagnostic test for diagnosing a cardiac, thrombotic disorder;

wherein the level and result assessed is indicative of a thrombotic event.

25. The method of claim 24, wherein the diagnostic test is an electrocardiogram.

26. The method of claim 25, further comprising assessing a level of at least one thrombin generation marker, wherein the level of the platelet activation marker, the level of the thrombin generation marker, and the result of the diagnostic test are indicative of the presence or absence of a thrombotic disorder.

27. The method of claim 26, wherein the thrombin generation marker is selected from a group consisting of creatine kinase muscle brain, D-Dimer, F1.2, thrombin anti-thrombin, soluble fibrin monomer, fibrin

peptide A, myoglobin, thrombin precursor protein, platelet monocyte aggregate and troponin.

28. The method of claim 27, further comprising assessing a level of at least two platelet activation markers.

29. The method of claim 28, wherein assessing the level of at least two platelet activation markers comprises:

a) assessing the level of membrane bound P-selectin in a sample comprising platelets from said patient;

b) assessing the level of soluble P-selectin in a sample comprising blood components from said patient.

30. A method for diagnosing the presence or absence of myocardial infarction or angina in a patient comprising:

a) assessing a level of membrane bound P-selectin in a sample comprising platelets from said patient;

b) assessing a level of soluble P-selectin in a sample comprising blood components from said patient;

c) assessing a result of at least one diagnostic test for diagnosing myocardial infarction or angina, and

d) assessing a level of a thrombin generation marker,

wherein the levels as assessed in steps a), b) and/or d) and/or a positive result as assessed in step (c) is indicative of a positive diagnosis.

31. The method of claim 30, wherein the diagnostic test is an electrocardiogram.

32. The method of claim 31, wherein the thrombin generation marker is selected from a group consisting of creatine kinase muscle brain, D-Dimer, F1.2, thrombin anti-thrombin, soluble fibrin monomer, fibrin peptide A, myoglobin, thrombin precursor protein, platelet monocyte aggregate and troponin.

33. An apparatus for diagnosing a thrombotic disorder in a patient comprising:

a) means for detecting the level of soluble P-selectin in a patient sample comprising blood components, and

b) means for detecting the level of membrane bound P-selectin in a patient sample comprising platelets.

34. An apparatus of claim 33, further comprising a means for detecting a level of a thrombin generation marker in a sample.

35. An apparatus of claim 34, further comprising means for comparing the levels detected in steps a) and b) to normal levels to obtain a result.

36. The apparatus of claim 35, further comprising means for displaying the results of said diagnosis.

37. An apparatus of claim 36, wherein the means for detecting the level of soluble P-selectin and the means for detecting membrane bound P-selectin is contained in a single chamber or capillary.

38. A kit for diagnosing the presence or absence of a thrombotic disorder in a patient comprising:

a) one or more reagents for detecting the level of membrane bound P-selectin in a patient sample comprising platelets, and

b) one or more reagents for detecting soluble P-selectin in a patient sample comprising aqueous blood components.

39. A kit of claim 38, further comprising one or more regents for detecting a level of a thrombin generation marker in a sample.

40. A kit of claim 39, wherein said one or more reagents for detecting soluble P-selectin are used for carrying out an enzyme-linked immunosorbent assay.

41. A kit of claim 40, wherein said one or more reagents for detecting membrane bound P-selectin are used for carrying out a flow cytometric analysis.

42. A kit of claim 40, wherein said one or more reagents for detecting membrane bound P-selectin are used for carrying out a radioimmunoassay.

43. A kit of claim 39, further comprising one or more reagents for detecting the number of platelets.

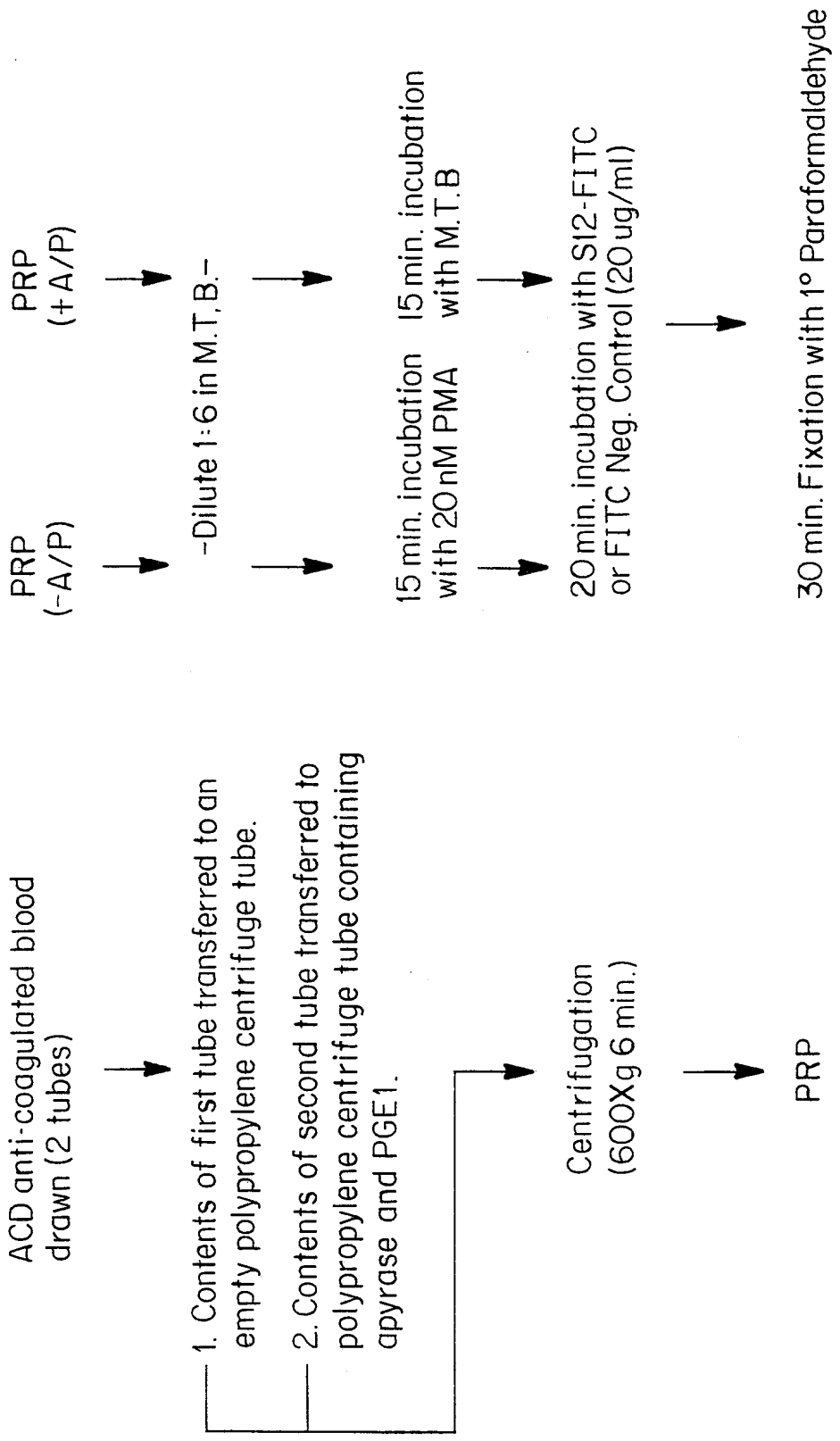
ACD anti-coagulated blood drawn (2 tubes)
1. Contents of first tube transferred to an empty polypropylene centrifuge tube.
2. Contents of second tube transferred to polypropylene centrifuge tube containing apyrase and PGE1.
Centrifugation (600Xg 6 min.)
PRP
PRP (-A/P)
PRP (+A/P)
-Dilute 1:6 in M.T.B.-
15 min. incubation with 20 nM PMA
15 min. incubation with M.T.B
20 min. incubation with SI2-FITC or FITC Neg. Control (20 ug/ml)
30 min. Fixation with 1° Paraformaldehyde

FIG. 1

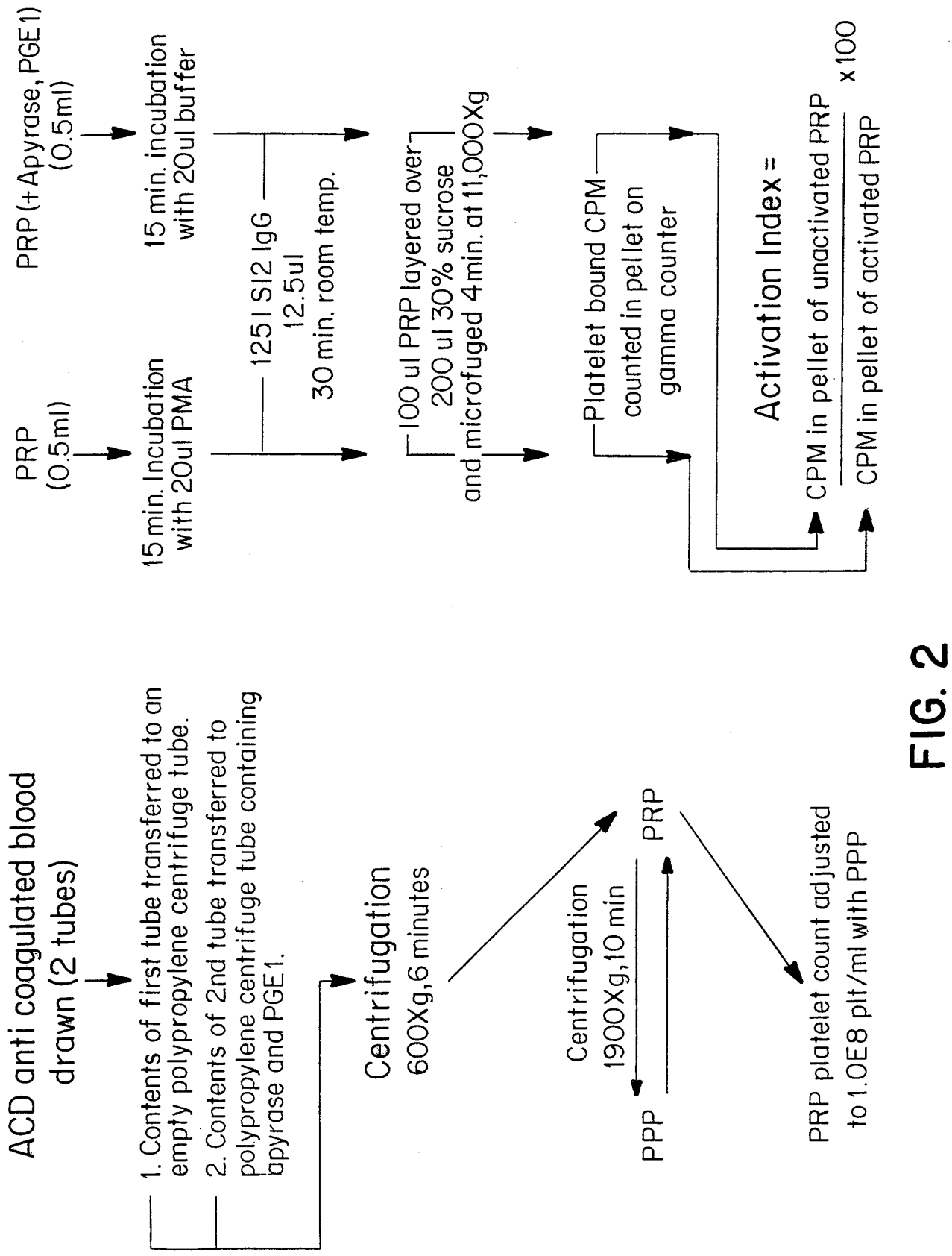
ACD anti coagulated blood drawn (2 tubes)
1. Contents of first tube transferred to an empty polypropylene centrifuge tube.
2. Contents of 2nd tube transferred to polypropylene centrifuge tube containing apyrase and PGE1.
Centrifugation 600Xg,6 minutes
Centrifugation 1900Xg,10 min
PPP
PRP
PRP platelet count adjusted to 1.0E8 plt/ml with PPP
PRP (0.5ml)
15 min. Incubation with 20ul PMA
PRP (+Apyrase, PGE1) (0.5ml)
15 min. incubation with 20ul buffer
125I SI2 IgG 12.5ul 30 min. room temp.
100 ul PRP layered over 200 ul 30% sucrose and microfuged 4min. at 11,000Xg
Platelet bound CPM counted in pellet on gamma counter
Activation Index =
CPM in pellet of unactivated PRP / CPM in pellet of activated PRP x100

FIG. 2

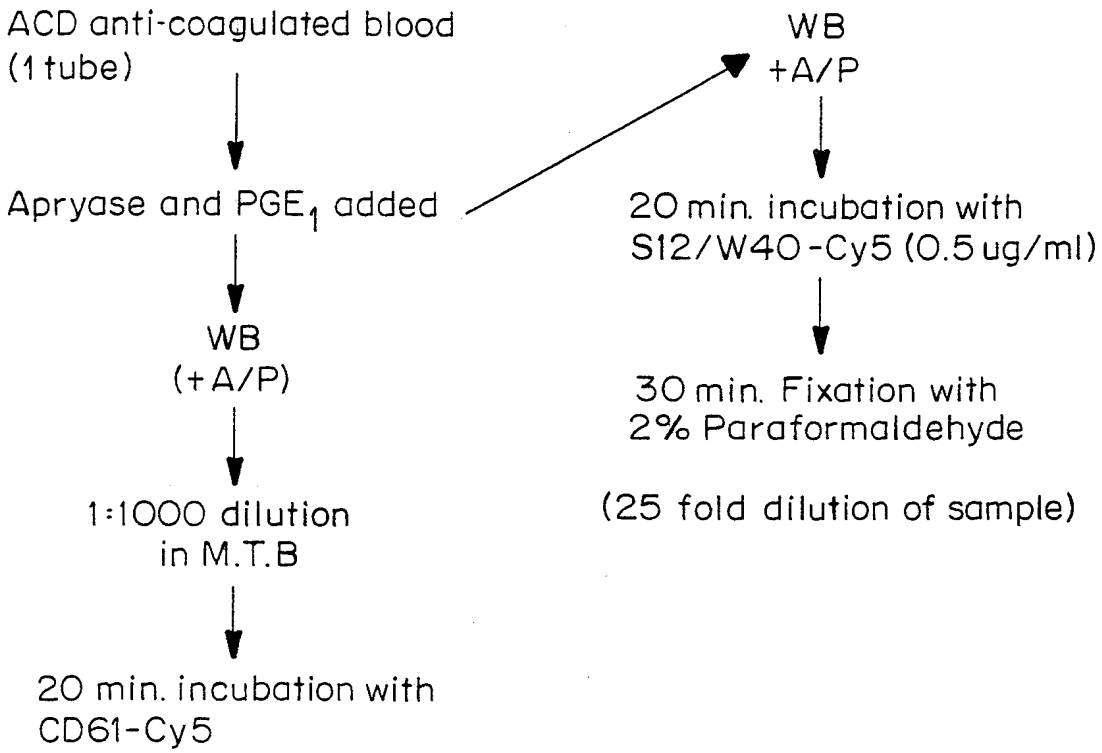
ACD anti-coagulated blood
(1 tube)
Apryase and PGE1 added
WB
(+A/P)
1:1000 dilution
in M.T.B
20 min. incubation with
CD61-Cy5
WB
+A/P
20 min. incubation with
S12/W40-Cy5 (0.5 ug/ml)
30 min. Fixation with
2% Paraformaldehyde
(25 fold dilution of sample)

FIG. 3

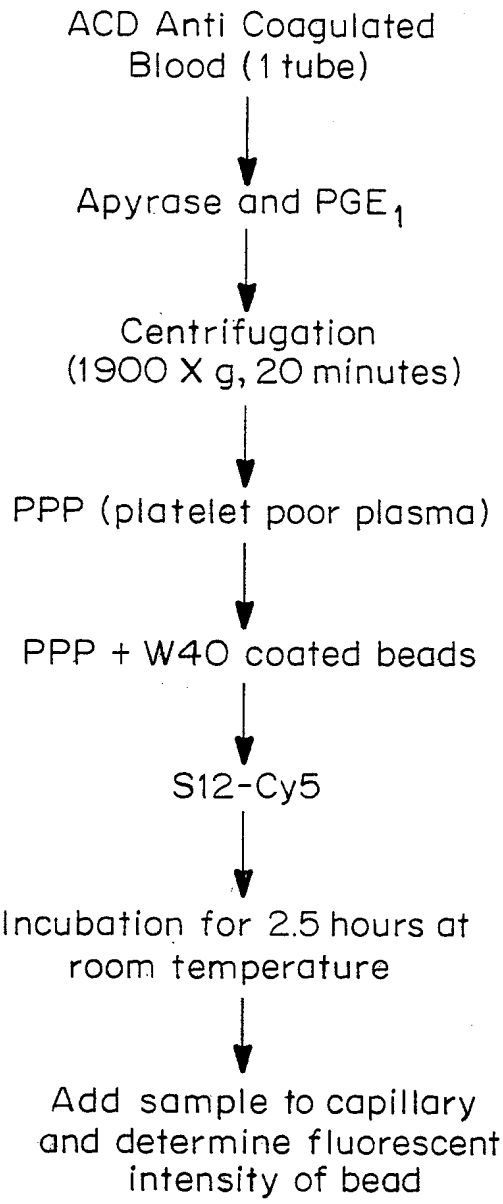
ACD Anti Coagulated Blood (1 tube)
Apyrase and PGE1
Centrifugation (1900 X g, 20 minutes)
PPP (platelet poor plasma)
PPP + W40 coated beads
S12-Cy5
Incubation for 2.5 hours at room temperature
Add sample to capillary and determine fluorescent intensity of bead

FIG. 4

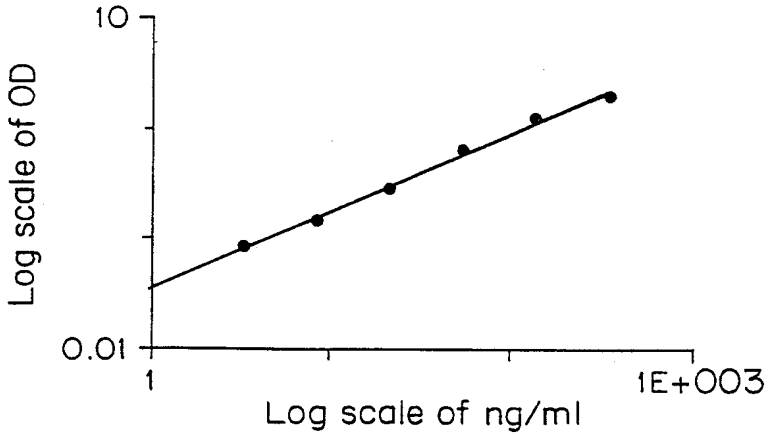

FIG. 5

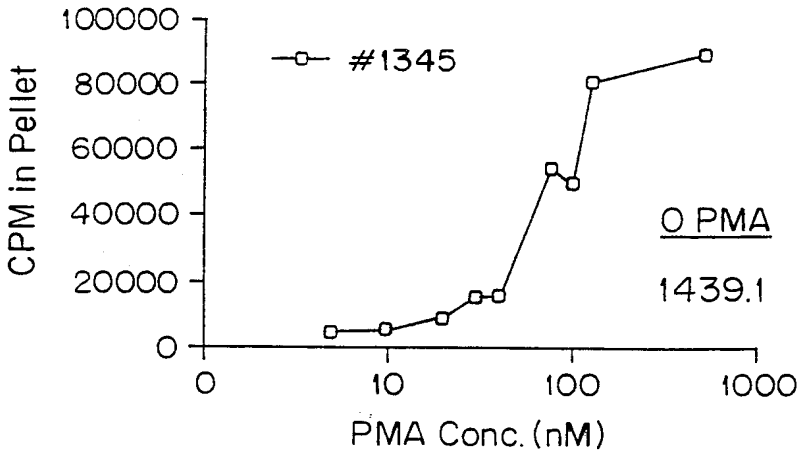

FIG. 6A

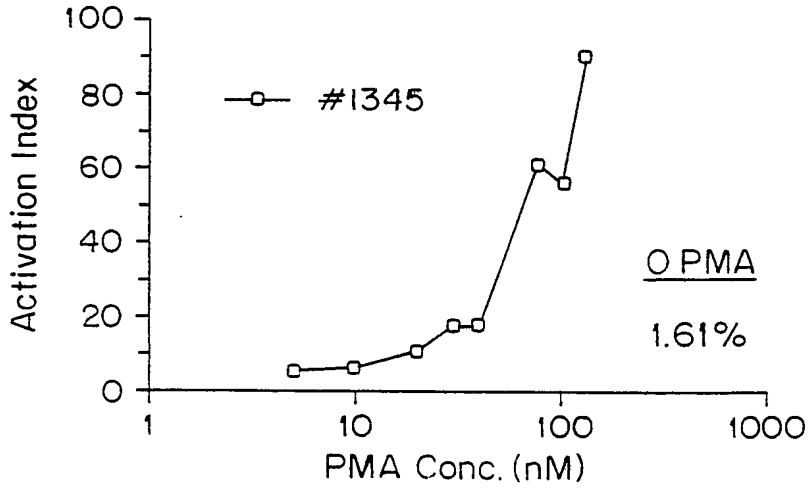

FIG. 6B

INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/US 98/21110

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 378 464 A (MCEVER RODGER P) 3 January 1995 see column 23, line 14 - line 18 see column 23, line 24 - line 30 ---	1-43
X	WO 94 25067 A (CYTEL CORP ;CHESNUT ROBERT W (US); POLLEY MARGARET J (US); PAULSON) 10 November 1994 see page 39, line 17 - page 40, line 23 see claim 8 ---	1,2
X	US 5 529 902 A (KOTTKE BRUCE A ET AL) 25 June 1996 see the whole document --- -/--	1,2

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 February 1999	09/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hoekstra, S

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INTERNATIONAL SEARCH REPORT

Inter ɔnal Application No
PCT/US 98/21110

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	DEHMER, G.J. ET AL.: "Assessment of platelet activation by cornoary sinus blood sampling during balloon angioplasty and directional coronary atherectomy" AMERICAN JOURAL OF CARDIOLOGY, vol. 80, no. 7, 1 October 1997, page 871-877 XP002092792 see page 871, left-hand column, line 1 ---	1,2
X	BLANN, A.D. ET AL.: "Soluble P-selectin in Atherosclerosis: A comparison with endothelial cell and platelet markers" THROMB. HAEMOST., vol. 77, no. 6, June 1997, pages 1077-1080, XP002092793 cited in the application see page 1080, left-hand column ---	1,2
X	ITOH, T. ET AL.: "Can the risk for acute cardiac events in acute coronary syndrome be indicated by platelet membrane activation marker P-selectin" PATHOPHYSIOLOGY AND NATURAL HISTORY, vol. 6, no. 8, August 1995, pages 645-650, XP002092794 cited in the application see the whole document ---	1,2
A,P	COLL-VINENT, B. ET AL.: "Circulating soluble adhesion molecules in patients with classical polyarteritis nodosa" BRITISH JOURNAL OF RHEUMATOLOGY, vol. 36, no. 11, November 1997, pages 1178-1183, XP002092795 see the whole document ---	3,4
X	CHONG, B.H. ET AL.,: "plasma P-selectin is increased in Thrombotic consumptive paltelet disorders" BLOOD, vol. 83, no. 6, 15 March 1995, pages 1535-1541, XP002092796 cited in the application see abstract see page 1540, right-hand column, line 14 - line 18 ---	3-5
P,X	SEREBRUANY, V.L. ET AL.: "Antecedent aspirin therapy inhibits baseline platelet activity in patients presenting with acute myocardial infarction." CORONARY CARE, vol. 90, no. 1, July 1998, pages 37-42, XP002092797 see abstract; table 2 -----	1-11

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ɔnal Application No
PCT/US 98/21110

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